PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, 5/08	A1	(11) International Publication Number: WO 99/57248 (43) International Publication Date: 11 November 1999 (11.11.99)

(21) International Application Number: PCT/US98/08716

(22) International Filing Date: 30 April 1998 (30.04.98)

(71) Applicant (for all designated States except US): PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): McMAHON, Andrew, P. [GB/US]; 128 Kendall Road, Lexington, MA 02173 (US). LEE, Scott, K. [US/US]; 54 Chestnut Street, Cambridge, MA 02139 (US). TAKADA, Shinji [-/-]; -

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: INDUCTION OF NEURONAL REGENERATION

(57) Abstract

An enriched population of mammalian dorsal neural progenitor cells, e.g., dopaminergic neural precursor cells, are described that are useful to induce neuronal regeneration in mammals suffering from a neurodegenerative disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

INDUCTION OF NEURONAL REGENERATION

Background of the Invention

The invention relates to neuronal growth and differentiation.

Wnt polypeptides are secreted cysteine-rich glycosylated polypeptides that play a role in the development of a wide range of organisms. The Wnt family of polypeptides contains at least 16 mammalian members which bind to an extracellular domain of a family of cell surface proteins called Frizzled receptors. Wnt polypeptides may play a role in embryonic induction, generation of cell polarity, and specification of cell fate. Deregulation of Wnt signalling has been linked to tumor development.

Summary of the Invention

The invention is based on the discovery that Wnt polypeptides regulate neuronal precursor cell fate, i.e., the type of neuron into which a precursor cell differentiates depends qualitatively on the Wnt signal it receives. For example, Wnt-1 specifies midbrain cell fate. In addition to regulation of cell type, Wnt polypeptides regulate neural precursor state, i.e., proliferation versus differentiation. A stem cell phenotype is characterized by mitotic activity and a lack of characteristics associated with a mature terminally-differentiated neuron, whereas a differentiated phenotype is characterized by a lack of proliferation and acquisition of properties, e.g., morphology or cell surface proteins, associated with a particular terminally-differentiated neural cell type.

The invention features an enriched population of mammalian dorsal neural precursor cells that maintain a stem cell phenotype in the presence of a Wnt polypeptide. By an "enriched population" is meant a population of

cells that has been treated with a Wnt polypeptide to selectively expand a desired neural precursor cell type. Thus, an enriched population of neural precursor cells is not naturally-occurring, but contains a higher concentration of neural precursor cells having a particular cell fate compared to the concentration in a naturally-occurring population of stem cells.

The Wnt polypeptide is preferably a Wnt-1 class polypeptide such as Wnt-1, Wnt-2, Wnt-3a, Wnt-7a, and Wnt-7b. A Wnt-1 class polypeptide is a Wnt polyeptide that transforms C57MG cells in culture. Other Wnt polypeptides, e.g., Wnt-5a, that play a role in midbrain development may also be used to culture stem cells.

A drawback of conventional stem cell preparations is that they heterogenous, i.e., a precursor cell with a particular cell fate may constitute only a small fraction of the population. The invention solves this problem by providing a method of selecting for a desired precursor cell type, i.e., by contacting the cell with a Wnt polypeptide. For example, the invention provides a method of treating a heterogeneous population of neural cell precursor cells to enrich for neural precursor cells committed to a desired cell fate by culturing the population with a Wnt polypeptide, e.g., a Wnt-1 class polypeptide. Neural precursor cells selectively proliferate in the presence of the Wnt polypeptide, whereas other precursor cells do not proliferate (or proliferate at a rate lower than that of the dorsal neural precursor cells). Thus, repeated culturing of the population in this manner yields a population of neural precursor cells that is progressively more enriched for dorsal neural precursor cells. The enriched population of dorsal neural precursor cells is at least 60%, preferably at least 75%, more preferably at least 80%, more preferably at least 90%, more preferably at least

95%, more preferably at least 98%, and most preferably 100% dorsal neural precursor cells.

For example, the invention encompasses an enriched population of mammalian dopaminergic neuron precursor cells. Selection of such cells is accomplished by contacting a heterogenous population of precursor cells with a Wnt-1 class polypeptide. The cells may be human or porcine cells and may be derived from fetal tissue. The cells are mitotically-active and maintaining a stem cell phenotype in the presence of a Wnt polypeptide. In the absence of a Wnt polypeptide, the cells cease proliferating and differentiate into dopaminergic neurons. A dopaminergic neuron is one that produces dopamine. Preferably, the Wnt polypeptide is human Wnt-1 or a fragment of Wnt-1 that is capable of stimulating proliferation of such cells and arresting differentiation. Since Wnt polypeptides have mitogenic activity for neural precursor cells, a method of stimulating cell proliferation of a dorsal neural precursor cell is carried out by contacting the cell in culture or *in vivo* with a Wnt-1 polypeptide and/or a Wnt-3a polypeptide. To promote proliferation of mammalian dopaminergic neuron precursor cells, the polypeptide preferably has a sequence that is at least 80% identical to amino acid sequence of naturally-occurring human Wnt-1 (SEQ ID NO:1) and has a biological property of naturally-occurring Wnt-1, e.g., the ability to maintain the neural stem cell phenotype of a neural precursor cell in culture.

Table 1: Human Wnt-1 amino acid sequence

```
      1 MGLWALLPGW VSATLLLALA ALPAALAANS SGRWWGIVNV ASSTNLLTDS
KSLQLVLEPS
     61 LQLLSRKQRR LIRQNPGILH SVSGGLQSAV RECKWQFRNR RWNCPTAPGP
HLFGKIVNRG
    121 CRETAFIFAI TSAGVTHSVA RSCSEGSIES CTCDYRRRGP GGPDWHWGGC
SDNIDFGRLF
    181 GREFVDSGEK GRDLRFLMNL HNNEAGRTTV FSEMRQECKC HGMSGSCTVR
TCWMRLPTLR
    241 AVGDVLRDRF DGASRVLYGN RGSNRASRAE LLRLEPEDPA HKPPSPHDLV
YFEKSPNFCT
    301 YSGRLGTAGT AGRACNSSSP ALDGCELLCC GRGHRTRTQR VTERCNCTFH
WCCHVSCRNC
    361 THTRVLHECL  (SEQ ID NO:1)
```

Table 2: Human Wnt-2 amino acid sequence

```
MNAPLGGIWLWLPLLLTWLTPEVNSSWWYMRATGGSSRVMCDNV
PGLVSSQRQLCHRHPDVMRAISQGVAEWTAECQHQFRQHRWNCNTLDRDHSLFGRVLL
RSSRESAFVYAISSAGVVFAITRACSQGEVKSCSCDPKKMGSAKDSKGIFDWGGCSDN
IDYGIKFARAFVDAKERKGKDARALMNLHNNRAGRKAVKRFLKQECKCHGVSGSCTLR
TCWLAMADFRKTGDYLWRKYNGAIQVVMNQDGTGFTVANERFKKPTKNDLVYFENSPD
YCIRDREAGSLGTAGRVCNLTSRGMDSCEVMCCGRGYDTSHVTRMTKCGCKFHWCCAV
RCQDCLEALDVHTCKAPKNADWTTAT  (SEQ ID NO:2)
```

Where a particular polypeptide or nucleic acid molecule is said to have a specific percent identity to a reference polypeptide or nucleic acid molecule of a defined length, the percent identity is relative to the reference polypeptide or nucleic acid molecule. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Sequence identity can be measured using sequence analysis software (for example, the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705), with the default parameters as specified therein.

An enriched population of mammalian dorsal hindbrain precursor cells is also within the invention. Such cells are selected by contacting a heterogenous population of cells with a mixture of a Wnt-1 polypeptide and a Wnt-3a polypeptide. An enriched population of mitotically-active mammalian hippocampal neuron precursor cells are selected by culturing the cells in the presence of a Wnt-1 class polypeptide such as Wnt-3a; the cells maintain a stem cell phenotype in culture in the presence of a Wnt-3a polypeptide. Such precursor cells cease proliferating and differentiate into hippocampal neurons in the absence of the Wnt-3a polypeptide. Preferably, the Wnt-3a polypeptide has a sequence that is at least 80% identical to SEQ ID NO:2 and has a biological property of naturally-occurring Wnt-3a, e.g., the ability to maintain a neural stem cell phenotype in culture.

<u>Table 3: Murine Wnt-3a amino acid sequence</u>

```
MAPLGYLLVLCSLKQALGSYPIWWSLAVGPQYSSLSTQPILCAS
IPGLVPKQLRFCRNYVEIMPSVAEGVKAGIQECQHQFRGRRWNCTTVSNSLAIFGPVL
DKATRESAFVHAIASAGVAFAVTRSCAEGSAAICGCSSRLQGSPGEGWKWGGCSEDIE
FGGMVSREFADARENRPDARSAMNRHNNEAGRQAIASHMHLKCKCHGLSGSCEVKTCW
WSQPDFRTIGDFLKDKYDSASEMVVEKHRESRGWVETLRPRYTYFKVPTERDLVYYEA
SPNFCEPNPETGSFGTRDRTCNVSSHGIDGCDLLCCGRGHNARTERRREKCHCVFHWC
CYVSCQECTRVYDVHTCK   (SEQ ID NO:3)
```

Table 10: Human Wnt-3a amino acid sequence

```
CKCHGLSGSC EVKTCWWSQP DFRAIGDFLK DKYDSASEMV VEKHRESRGW
VETLRPRYTY FKVPTERDLV YYEASPNFCE PNPETGSFGT RDRTCNVSSH
GIDGCDLLCC GRGHNARAER RREKCRCVFH WCC   (SEQ ID NO:10)
```

<u>Table 4: Human Wnt-7a amino acid sequence</u>

```
  1 MNRKALRCLG HLFLSLGMVC LRIGGFSSVV ALGATIICNK IPGLAPRQRA ICQSRPDAII
 61 VIGEGSQMGL DECQFQFRNG RWNCSALGER TVFGKELKVG SRDGAFTYAI IAAGVAHAIT
121 AACTHGNLSD CGCDKEKQGQ YHRDEGWKWG GCSADIRYGI GFAKVFVDAR EIKQNARTLM
181 NLHNNEAGRK ILEENMKLEC KCHGVSGSCT TKTCWTTLPQ FRELGYVLKD KYNEAVHVEP
241 VRASRNKRPT FLKIKKPLSY RKPMDTDLVY IEKSPNYCEE DPVTGSVGTQ GRACNKTAPQ
```

301 ASGCDLMCCG RGYNTHQYAR VWQCNCKFHW CCYVKCNTCS ERTEMYTCK

Table 5: Human Wnt-7b partial amino acid sequence

```
  1 GVSGSCTTKT CWTTLPKFRE VGHLLKEKYN AAVQVEVVRA SRLRQPTFLR IKQLRSYQKP
 61 METDLVYIEK SPNYCEEDAA TGSVGTQGRI CNRTSPGADG CDTMCCGRGY NTHQYTKVWQ
121 CNCK (SEQ ID NO:5)
```

Table 6: Human Wnt-5a amino acid sequence

```
  1 MAGSAMSSKF FLVALAIFFS FAQVVIEANS WWSLGMNNPV QMSEVYIIGA QPLCSQLAGL
 61 SQGQKKLCHL YQDHMQYIGE GAKTGIKECQ YQFRHRRWNC STVDNTSVFG RVMQIGSRET
121 AFTYAVSAAG VVNAMSRACR EGELSTCGCS RAARPKDLPR DWLWGGCGDN IDYGYRFAKE
181 FVDARERERI HAKGSYESAR ILMNLHNNEA GRRTVYNLAD VACKCHGVSG SCSLKTCWLQ
241 LADFRKVGDA LKEKYDSAAA MRLNSRGKLV QVNSRFNSPT TQDLVYIDPS PDYCVRNEST
301 GSLGTQGRLC NKTSEGMDGC ELMCCGRGYD QFKTVQTERC HCKFHWCCYV KCKKCTEIVD
361 QFVCK (SEQ ID NO:6)
```

Other patterning signals, e.g., Bmp polypeptides or Hedgehog polypeptides, are also used to induce differentiation of an enriched population of neural precursor cells into a differentiated neural cell type.

An population of neural precursor cells that is enriched for a particular type of precursor cell is useful clinically, e.g., to repopulate a depleted population of a particular type of neuron. Depletion of subpopulations of neurons may be the result of the progression of a neurodegenerative disease such as Parkinson's Disease, Amyotrophic Lateral Sclerosis, Diffuse Lewy Body Disease, Cortical-basal Ganglionic Degeneration, Hallervorden-Spatz Disease, or Myoclonic Epilepsy. A method of inducing neuronal regeneration in an adult mammal suffering from a neurodegenerative disorder is carried out by transplanting into the affected mammal an enriched population of dorsal neural precursor cells such as that cultured in the presence of one or more Wnt polypeptides. To promote proliferation of the transplanted stem cells *in vivo*, the method may also include a step of administering to the mammal a Wnt polypeptide or Wnt agonist systemically or locally at the site of transplantation. For example, a patient suffering from Parkinson's disease is treated by transplanting into the brain of the patient an enriched

population of dopaminergic neuron precursor cells. A Wnt-1 polypeptide may be administered concurrently or subsequent to transplantation.

The invention also includes a transgenic non-human mammal, e.g., a rodent such as a mouse, the germ cells and somatic cells of which contain a null mutation, e.g., a deletion, in DNA encoding a Wnt polypeptide. These animals can serve as useful models of neural development. By "null mutation" is meant an alteration in the nucleotide sequence that renders the gene incapable of expressing a functional protein product. The mutation could be in a Wnt gene regulatory region or in the coding sequence. It can, e.g., introduce a stop codon that results in production of a truncated, inactive gene product or it can be a deletion of all or a substantial portion of the coding sequence.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The invention provides methods of selecting for neural precursor cells that will differentiate into a particular type of neuron upon exposure to a differentiation-inducing condition or composition and methods for growing such precursor cells in culture. The methods permit expansion of precursor cells of a desired cell fate to achieve large number of cells suitable for clinical transplantation.

Neural Stem Cells

Primary neural progenitor cells are obtained from a mammalian source, e.g., fetal CNS precursor tissue such as developing neural crest tissue, using known methods. Such primary cells are cultured in the presence of a Wnt polypeptide such as Wnt-1 class polypeptide (purified from a natural source or produced recombinantly) in

conventional tissue culture medium such as Dulbecco's Modified Eagles Medium (DMEM) containing fetal calf serum or in serum-free tissue culture medium.

Wnt polypeptides regulate neuronal precursor cell fate as well as neural precursor state. Wnt polypeptides that belong to the Wnt-1 class of Wnt polypeptides are preferably used to culture neural precursor cells for the purpose of maintaining a stem cell phenotype and promoting proliferation. A Wnt-1 class polypeptide is a Wnt polyeptide and that transforms C57MG cells in culture. To determine whether a Wnt polypeptide is a Wnt-1 class polypeptide, C57MG cells (an epithelial cell line derived from normal mouse mammary tissue) are cultured in the presence and absence of the Wnt polypeptide using known methods, e.g., that described by Wong et al., 1994, Mol. Cell. Biol. 14:6278-6286, and their morphology assessed for a transformed phenotype. Normal C57MG cells grow in a monolayer with a regular, cuboidal appearance at confluence, whereas culturing C57MG cells in the presence of a Wnt-1 class polypeptide causes the cells to become transformed, i.e., refractile and elongated, growing over other cells in a disorganized manner. Wnt polypeptides of the Wnt-1 class cause C57MG cells to assume a transformed phenotype. Human Wnt polypeptides which belong to the Wnt-1 class include Wnt-1 (GENBANK Accession #139743, Wnt-2 (GENBANK Accession #139750), Wnt-3a, Wnt-7a (GENBANK Accession #2501663), and Wnt-7b (GENBANK Accession #546573). A Wnt polypeptide, e.g., human Wnt-5a (GENBANK Accession #731157), that is not a member of the Wnt-1 class may also be used (with or without a Wnt-1 class polypeptide) to culture neural precursor cells.

The cells are cultured in the presence or absence of feeder cells. Feeder cells may be engineered to produce a recombinant Wnt-1 class polypeptide such as

Wnt-1 and/or Wnt-3a, e.g., by introducing DNA encoding a Wnt polypeptide, e.g., DNA encoding Wnt-1, Wnt-2, Wnt-3a, Wnt-7a or Wnt-7b, into the cell and culturing the cell under conditions that permit expression of the recombinant polypeptide and secretion of the polypeptide into the extracellular environment. For example, feeder cells can be transfected with an expression vector containing DNA having the sequence of naturally-occuring Wnt-1, Wnt-2, or Wnt-3a.

Table 7: Human Wnt-1 Nucleotide Sequence

```
      1 atgtatgtat gtatgtatgt atgtatgtat acgtgcgtgc acctgtgtgt
gcttggtgtc
     61 agtggggctc agacatcacc tgattccctg gaactggagt tacaggtggc
tataagccac
    121 cacttgggtg ctgagaacag agtccgggcc tctggcagag cagtcagtgc
ttttagccac
    181 tgagccactc tcatcccccc aattatgttc atcttgagtt gggcaggtac
ggtggcggaa
    241 taggcctgta atcccagcag tcactggacc atcatgggtt ctacatatta
aacctttatg
    301 ttaggtaggg tcacacagca agatccggtc acaaaaccag caacaacaaa
aaccaaaagg
    361 agccagcttc ttcccacaag cattctttcc ctcaggtctt cagctccatc
tgacagctac
    421 tcggctggtg gtcctatcct ttctgagcct agttgccaga gaaacaagcc
cggttcatct
    481 tcatgactag cacatctaat gataagcaca ggttgactca aggtgccata
gagtgacact
    541 aggtacccag agcgacagaa tgacacctat gagtgcacgt cgttaatcac
aaacacacac
    601 acacacacac acacacacac acacacacac tcatgcaccc acctgcaaac
acaattgcag
    661 ccttctggac gtctcctgtc acagccccac ctccttcctg atacactgcg
ttaagtggtg
    721 actgtaacaa aatgacttca tgctctccct gtcctgagcc aaattacaca
attatttgga
    781 aagggctcaa aatgttcttc gttagaagtt tctggataca ccaatacaca
ggagcgtgca
    841 ccctcagaac acatgtacac tttgacttaa tctcacgggt gacacaccga
cgcttacact
    901 ccccctagcc cacagaggca aactgctggg cgcttctgag tttctcactg
ccaccagctc
    961 ggtttgctca gcctaccccc gcaccccgcg cccgggaatc cctgaccaca
gctccaccca
   1021 tgctctgtct ccttcttttc cttctctgtc cagccgtcgg ggttcctggg
tgaggaagtg
   1081 tctccacgga gtcgctggct agaaccacaa ctttcatcct gccattcaga
atagggaaga
   1141 gaagagacca cagcgtaggg gggacagagg agacggactt cgagaggaca
gccccaccgg
   1201 cgcgtgtggg ggaggcaatc caggctgcaa acaggttgtc cccagcgcat
tgtccccgcg
   1261 ccccctggcg gatgctggtc cccgacgggc tccggacgcg cagaagagtg
aggccggcgc
   1321 gcgtgggagg ccatcccaag gggaggggtc ggcggccagt gcagacctgg
aggcggggcc
```

1381 accaggcagg gggcgggggt gagccccgac ggttagcctg tcagctcttt gctcagaccg
1441 gcaagagcca cagcttcgct cgccactcat tgtctgtggc cctgaccagt gcgccctggt
1501 gcttttagtg ccgcccgggc ccggaggggc agcctcttct cactgcagtc agcgccgcaa
1561 ctataagagg cctataagag gcggtgcctc ccgcagtggc tgcttcagcc cagcagccag
1621 gacagcgaac catgctgcct gcggcccgcc tccagactta ttagagccag cctgggaact
1681 cgcatcactg ccctcaccgc tgtgtccagt cccaccgtcg cggacagcaa ccacagtcgt
1741 cagaaccgca gcacagaacc agcaaggcca ggcaggccat ggggctctgg gcgctgctgc
1801 ccagctgggt ttctactacg ttgctactgg cactgaccgc tctgcccgca gccctggctg
1861 ccaacagtag tggccgatgg tggtaagtga gctagtacgg ggtccgccac ttgtcctggg
1921 gcaaagagcc aggcacgggc cttacccagc tcccacgctg tggggatcac caacctacag
1981 accccctcg tgcattgtga cttcacatcc agggtgctca cacctagaac tagctctgct
2041 gaagtggggc acatcattgg catgcagaag cccagataca ccaggctcag agaccattcc
2101 catttaatac gaccccgttt ctgctgagca acaggtccca acctcgctgt ggtgggtgct
2161 caggtgtccc ttaggtcttg aaccaaaaaa aaaaaaaaaa aaaaaaaaaa accagatatt
2221 agctttgagg tgagggagtg gaattcctaa gtttttcaag gtgggcaagg ctgcaggtgg
2281 ggtttctcct cgggggctga cttgaagaaa ggaagagcta aggtagccat gccttttctg
2341 tccactcact agactctgga gctcagggcc aggcaaggat agggtggtac agcctgtatg
2401 gttaggatgc aggtcccctc ccctggactg aacccttatg catcccgcca ggggcatcgt
2461 gaacatagcc tcctccacga acctgttgac ggattccaag agtctgcagc tggtgctcga
2521 gcccagtctg cagctgctga gccgcaagca gcggcgactg atccgacaga acccggggat
2581 cctgcacagc gtgagtggag ggctccagag cgctgtgcga gagtgcaaat ggcaattccg
2641 aaaccgccgc tggaactgcc ccactgctcc ggggccccac ctcttcggca agatcgtcaa
2701 ccgaggtggg tgcccaggaa agcgacgctt ccgggattaa gggaaaagca gggtcatctc
2761 cagggcatag gcgggcgaag gcagggaaga catcccaggg ttatatgtga tcaaactgag
2821 aatcgcctgg tgccggcagt taccgtaggt cagcaccaga ttctttctag ccttgcgttg
2881 tgagcatgat ctttaacgtt gctggccact ggcccacaga aagggaattc cggatcgtgg
2941 gcgctgggcg acagctgttt ttccctagcc ttcctcaaag gtacctggga agctgatctc
3001 tgagggctag ctagggttgt gcttcgcacc cagcaaagtt tgcactgcca atactagtag
3061 cgatcttggc tatgcagatt tgttctactt gggaatctcc ccttggagct gctctgctag
3121 ggctctggag tctcagtaaa gcttagagag gagggcattc catgcttcgc acacatgact
3181 ccaaggatgt tggactgtag ggtaccaagt cttccaaaca gggtgctgag ttggccccac
3241 gccttctctc aactgatgcg ggtcgcttc acccacaggc tgccgagaaa cagcgttcat
3301 cttcgcaatc acctccgccg ggtcacaca ttccgtggcg cgctcctgct ccgaaggctc

3361 catcgagtcc tgcacctgcg actaccggcg gcgcggccct gggggccccg actggcactg
3421 ggggggctgc agtgacaaca tcgattttgg tcgcctcttt ggccgagagt tcgtggactc
3481 cggggagaag gggcgggacc tacgcttcct catgaacctt cacaacaacg aggcagggcg
3541 aacggtacgt cggtgtgtcc ggaaccaatg gcaggggaga tgtaagacag gtgcacgggg
3601 acagaggcac agggaggggc ttcccgagag agtgggactc taggagggaa gacagagaag
3661 aggtggtggt tgagggcaaa gaggttcctg agctgatgac agaacagaag agattagcag
3721 gctatcaaca cgtgggatgt attgagatgg ctccatggca cacttttgaa agataaaagt
3781 gacttgctgg cgtggagcag agtctggccg aatgtcccta tctcagcggg ccattttgca
3841 cttcctctct cccgagctta gtcacacctg gaccttggct gaagtttcca cagcatcgac
3901 gtgacccggg tggggtgggg gtggggaagt atgggtggtg gttcgtggga tgttggcttt
3961 gaccttttct tccctcctcc cctcgtcccc tcctccccca gaccgtgttc tctgagatgc
4021 gccaagagtg caaatgccac gggatgtccg gctcctgcac ggtgcgcacg tgttggatgc
4081 ggctgcccac gctgcgcgct gtgggcgacg tgctgcgcga ccgcttcgac ggcgcctccc
4141 gcgtccttta cggcaaccga ggcagcaacc gcgcctcgcg ggcggagctg ctgcgcctgg
4201 agcccgaaga ccccgcgcac aagcctccct cccctcacga cctcgtctac ttcgagaaat
4261 cgcccaactt ctgcacgtac agtggccgcc tgggcacagc tggcacagct ggacgagctt
4321 gcaacagctc gtctcccgcg ctggacggct gtgagctgct gtgctgtggc cgaggccacc
4381 gcacgcgcac gcagcgcgtc acggagcgct gcaactgcac cttccactgg tgctgccacg
4441 tcagctgccg caactgcacg cacacgcgcg ttctgcacga gtgtctatga ggtgccgcgc
4501 ctccgggaac gggaacgctc tcttccagtt ctcagacaca ctcgctggtc ctgatgtttg
4561 cccaccctac cgcgtccagc cacagtccca gggttcatag cgatccatct ctcccacctc
4621 ctacctgggg actcctgaaa ccacttgcct gagtcggctc gaaccctttt gccatcctga
4681 gggccctgac ccagcctacc tccctccctc tttgagggag actccttttg cactgccccc
4741 caatttggcc agagggtgag agaaagattc ttcttctggg gtgggggtgg ggaggtcaac
4801 tcttgaaggt gttgcggttc ctgatgtatt ttgcgctgtg acctctttgg gtattatcac
4861 ctttccttgt ctctcgggtc cctataggtc ccttgagttc tctaaccagc acctctgggc
4921 ttcaaggcct ttcccctccc acctgtagct gaagagtttc cgagttgaaa gggcacggaa
4981 agctaagtgg gaaaggaggt tgctggaccc agcagcaaaa ccctacattc tccttgtctc
5041 tgcctcggag ccattgaaca gctgtgaacc atgcctccct cagcctcctc ccaccccttc
5101 ctgtcctgcc tcctcatcac tgtgtaaata atttgcaccg aaatgtggcc gcagagccac
5161 gcgttcggtt atgtaaataa aactatttat tgtgctgggt tccagcctgg gttgcagaga
5221 ccaccctcac cccacctcac tgctcctctg ttctgctcgc cagtcctttt gttatccgac
5281 ctttttctc ttttacccag cttctcatag gcgcccttgc ccaccggatc agtatttcct

5341 tccactgtag ctattagtgg ctcctcgccc ccaccaatgt agtatcttcc tctgaggaat
5401 aaaatatcta tttttatcaa cgactctggt ccttgaatcc agaacacagc atggcttcca
5461 acgtcctctt cccttccaat ggacttgctt ctcttctcat agccaaacaa aagagataga
5521 gttgttgaag atctcttttc cagggcctga gcaaggaccc tgagatcctg accettggat
5581 gaccctaaat gagaccaact agggatc (SEQ ID NO:7)

Table 8: Human Wnt-2 Nucleotide Sequence

1 agcagagcgg acgggcgcgc gggaggcgcg cagagctttc gggctgcagg cgctcgctgc
61 cgctggggaa ttgggctgtg ggcgaggcgg tccgggctgg cctttatcgc tcgctgggcc
121 catcgtttga aactttatca gcgagtcgcc actcgtcgca ggaccgagcg gggggcgggg
181 gcgcggcgag gcggcggccg tgacgaggcg ctcccggagc tgagcgcttc tgctctgggc
241 acgcatggcg cccgcacacg gagtctgacc tgatgcagac gcaagggggt taatatgaac
301 gcccctctcg gtggaatctg gctctggctc cctctgctct tgacctggct cacccccgag
361 gtcaactctt catggtggta catgagagct acaggtggct cctccagggt gatgtgcgat
421 aatgtgccag gcctggtgag cagccagcgg cagctgtgtc accgacatcc agatgtgatg
481 cgtgccatta gccagggcgt ggccgagtgg acagcagaat gccagcacca gttccgccag
541 caccgctgga attgcaacac cctggacagg gatcacagcc ttttggcag ggtcctactc
601 cgaagtagtc gggaatctgc ctttgtttat gccatctcct cagctggagt tgtatttgcc
661 atcaccaggg cctgtagcca aggagaagta aaatcctgtt cctgtgatcc aaagaagatg
721 ggaagcgcca aggacagcaa aggcattttt gattggggtg gctgcagtga taacattgac
781 tatgggatca aatttgcccg cgcatttgtg gatgcaaagg aaaggaaagg aaaggatgcc
841 agagccctga tgaatcttca caacaacaga gctggcagga aggctgtaaa gcggttcttg
901 aaacaagagt gcaagtgcca cggggtgagc ggctcatgta ctctcaggac atgctggctg
961 gccatggccg acttcaggaa aacgggcgat tatctctgga ggaagtacaa tggggccatc
1021 caggtggtca tgaaccagga tggcacaggt ttcactgtgg ctaacgagag gtttaagaag
1081 ccaacgaaaa atgacctcgt gtattttgag aattctccag actactgtat cagggaccga
1141 gaggcaggct ccctgggtac agcaggccgt gtgtgcaacc tgacttcccg ggcatggac
1201 agctgtgaag tcatgtgctg tgggagaggc tacgacacct cccatgtcac ccggatgacc
1261 aagtgtgggt gtaagttcca ctggtgctgc gccgtgcgct gtcaggactg cctggaagct
1321 ctggatgtgc acacatgcaa ggcccccaag aacgctgact ggacaaccgc tacatgaccc
1381 cagcaggcgt caccatccac cttcccttct acaaggactc cattggatct gcaagaacac
1441 tggacctttg ggttctttct ggggggatat ttcctaaggc atgtggcctt tatctcaacg
1501 gaagcccct cttcctccct gggggcccca ggatgggggg ccacacgctg cacctaaagc
1561 ctaccctatt ctatccatct cctggtgttc tgcagtcatc tcccctcctg gcgagttctc
1621 tttggaaata gcatgacagg ctgttcagcc gggagggtgg tgggcccaga ccactgtctc
1681 cacccacctt gacgtttctt ctttctagag cagttggcca agcagaaaaa aaagtgtctc
1741 aaaggagctt tctcaatgtc ttcccacaaa tggtcccaat taagaaattc catacttctc
1801 tcagatggaa cagtaaagaa agcagaatca actgcccctg acttaacttt aacttttgaa
1861 aagaccaaga cttttgtctg tacaagtggt tttacagcta ccacccttag ggtaattggt
1921 aattacctgg agaagaatgg ctttcaatac ccttttaagt ttaaaatgtg tattttcaa
1981 ggcatttatt gccatattaa aatctgatgt aacaaggtgg ggacgtgtgt cctttggtac
2041 tatggtgtgt tgtatctttg taagagcaaa agcctcagaa agggattgct ttgcattact
2101 gtccccttga tataaaaaat ctttagggaa tgagagttcc ttctcactta gaatctgaag
2161 ggaattaaaa agaagatgaa tggtctggca atattctgta actattgggt gaatatggtg
2221 gaaaataatt tagtggatgg aatatcagaa gtatatctgt acagatcaag aaaaaaagga
2281 agaataaaat tcctatatca t (SEQ ID NO:8)

Table 9: Murine Wnt-3A Nucleotide Sequence

1 gaattcatgt cttacggtca aggcagaggg cccagcgcca ctgcagccgc gccacctccc
61 agggccgggc cagcccaggc gtccgcgctc tcggggtgga ctccccccgc tgcgcgctca
121 agccggcgat ggctcctctc ggatacctct tagtgctctg cagcctgaag caggctctgg
181 gcagctaccc gatctggtgg tccttggctg tgggacccca gtactcctct ctgagcactc
241 agcccattct ctgtgccagc atcccaggcc tggtaccgaa gcagctgcgc ttctgcagga

301 actacgtgga gatcatgccc agcgtggctg agggtgtcaa agcgggcatc caggagtgcc
361 agcaccagtt ccgaggccgg cgttggaact gcaccaccgt cagcaacagc ctggccatct
421 ttggccctgt tctggacaaa gccacccggg agtcagcctt tgtccatgcc atcgcctccg
481 ctggagtagc tttcgcagtg acacgctcct gtgcagaggg atcagctgct atctgtgggt
541 gcagcagccg cctccagggc tccccaggcg agggctggaa gtggggcggc tgtagtgagg
601 acattgaatt tggaggaatg gtctctcggg agtttgccga tgccagggag aaccggccgg
661 atgcccgctc tgccatgaac cgtcacaaca atgaggctgg gcgccaggcc atcgccagtc
721 acatgcacct caagtgcaaa tgccacgggc tatctggcag ctgtgaagtg aagacctgct
781 ggtggtcgca gccggacttc cgcaccatcg ggatttcct caaggacaag tatgacagtg
841 cctcggagat ggtggtagag aaacaccgag agtctcgtgg ctgggtggag accctgaggc
901 cacgttacac gtacttcaag gtgccgacag aacgcgacct ggtctactac gaggcctcac
961 ccaacttctg cgaacctaac cccgaaaccg gctccttcgg gacgcgtgac cgcacctgca
1021 atgtgagctc gcatggcata gatgggtgcg acctgttgtg ctgcgggcgc gggcataacg
1081 cgcgcactga gcgacggagg gagaaatgcc actgtgtttt ccattggtgc tgctacgtca
1141 gctgccagga gtgcacacgt gtctatgacg tgcacacctg caagtaggag agctcctaac
1201 acgggagcag ggttcattcc gaggggcaag gttcctacct gggggcgggg ttcctacttg
1261 gaggggtctc ttacttgggg actcggttct tacttgaggg cggagatcct acctgtgagg
1321 gtctcatacc taaggacccg gtttctgcct tcagcctggg ctcctatttg ggatctgggt
1381 tcctttttag gggagaagct cctgtctggg atacgggttt ctgcccgagg gtggggctcc
1441 acttggggat ggaattccaa tttgggccgg aagtcctacc tcaatggctt ggactcctct
1501 cttgacccga cagggctcaa atggagacag gtaagctact ccctcaacta ggtggggttc
1561 gtgcggatgg gtgggagggg agagattagg gtccctcctc ccagaggcac tgctctatct
1621 agatacatga gagggtgctt cagggtgggc cctatttggg cttgaggatc ccgtgggggc
1681 ggggcttcac cccgactggg tggaactttt ggagaccccc ttccactggg gcaaggcttc
1741 actgaagact catgggatgg agctccacgg aaggaggagt tcctgagcga gcctgggctc
1801 tgagcaggcc atccagctcc catctggccc ctttccagtc ctggtgtaag gttcaacctg
1861 caagcctcat ctgcgcagag caggatctcc tggcagaatg aggcatggag aagaactcag
1921 gggtgatacc aagacctaac aaaccccgtg cctgggtacc tcttttaaag ctctgcaccc
1981 cttcttcaag ggctttccta gtctccttgg cagagctttc ctgaggaaga tttgcagtcc
2041 cccagagttc aagtgaacac ccatagaaca gaacagactc tatcctgagt agagagggtt
2101 ctctaggaat ctctatgggg actgctagga aggatcctgg gcatgacagc ctcgtatgat
2161 agcctgcatc cgctctgaca cttaatactc agatctcccg ggaaacccag ctcatccggt
2221 ccgtgatgtc catgccccaa atgcctcaga gatgttgcct cactttgagt tgtatgaact

2281 tcggagacat ggggacacag tcaagccgca gagccagggt tgtttcagga cccatctgat
2341 tccccagagc ctgctgttga ggcaatggtc accagatccg ttggccacca ccctgtcccg
2401 agcttctcta gtgtctgtct ggcctggaag tgaggtgcta catacagccc atctgccaca
2461 agagcttcct gattggtacc actgtgaacc gtccctcccc ctccagacag gggaggggat
2521 gtggccatac aggagtgtgc ccggagagcg cggaaagagg aagagaggct gcacacgcgt
2581 ggtgactgac tgtcttctgc ctggaacttt gcgttcgcgc ttgtaacttt attttcaatg
2641 ctgctatatc cacccaccac tggatttaga caaaagtgat ttcttttttt tttttttctt
2701 ttctttctat gaaagaaatt attttagttt atagtatgtt tgtttcaaat aatggggaaa
2761 gtaaaaagag agaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaa
(SEQ ID NO:9)

<u>Table 11: Human Wnt-3a nucleotide sequence</u>

tgtaagtgcc acgggctgtc gggcagctgc gaggtgaaga catgctggtg
gtcgcaaccc gacttccgcg ccatcggtga cttcctcaag gacaagtacg
acagcgcctc ggagatggtg gtggagaagc accgggagtc ccgcggctgg
gtggagaccc tgcggccgcg ctacacctac ttcaaggtgc ccacggagcg
cgacctggtc tactacgagg cctcgcccaa cttctgcgag cccaaccctg
agacgggctc cttcggcacg cgcgaccgca cctgcaacgt cagctcgcac
ggcatcgacg gctgcgacct gctgtgctgc ggccgcggcc acaacgcgcg
agcggagcgg cgccgggaga agtgccgctg cgtgtttcac tggtgctgt
(SEQ ID NO:11)

Stem cells may be obtained from a a heterologous donor animal such as a pig. The animal is euthanized and tissue removed using a sterile procedure. Brain areas of particular interest include any area from which progenitor cells can be obtained which will serve to restore function to a degenerated area of the host's brain. These regions include areas of the CNS including the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the peripheral nervous system (PNS) including the carotid body and the adrenal medulla. For example, cells may be obtained from the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, or the substantia nigra pars compacta (which is found to be degenerated in Parkinson's Disease patients).

Human heterologous neural progenitor cells may be derived from fetal tissue obtained from elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, in particular during epilepsy surgery, and more particularly during temporal lobectomies and hippocampalectomies.

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Dissociation can be obtained using any known procedure, including treatment with enzymes, e.g., trypsin or collagenase, or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose. Low Ca^{2+} aCSF contains the same ingredients except for $MgCl_2$ at a concentration of 3.2 mM and $CaCl_2$ at a concentration of 0.1 mM.

Dissociated cells can be placed into any culture medium capable of supporting cell growth, including MEM, DMEM, RPMI, F-12. The medium may containin supplements which support cellular metabolism such as glutamine and other amino acids, vitamins, minerals and proteins such as transferrin. In some cases, the medium may contain bovine, equine, chicken or human serum. A preferable medium for neural precursor cells is a mixture of DMEM and F-12. Conditions for culturing mimic physiological conditions, e.g., physiological pH, preferably between pH 6-8, more preferably close to pH 7, even more particularly about pH 7.4 at a temperature that is at or close to physiological temperature.

Cells can be grown in suspension or on a fixed substrate, but proliferation of the precursor cells is preferably done in suspension to generate large numbers of cells by formation of "neurospheres" (see, for example, Reynolds et al., 1992, Science 255:1070-1709; and PCT Publications WO93/01275, WO94/09119, WO94/10292, and WO94/16718). Cell suspensions in culture medium are supplemented with any growth factor which allows for the proliferation of precursor cells and seeded in any receptacle capable of sustaining cells, preferably in culture flasks or roller bottles. Cells typically proliferate within 3-4 days in a 37°C incubator, and proliferation can be reinitiated at any time after that by dissociation of the cells and resuspension in fresh medium containing growth factors.

In the absence of substrate, cells lift off the floor of the flask and continue to proliferate in suspension forming a hollow sphere of undifferentiated cells. After approximately 3-10 days *in vitro*, the proliferating clusters (neurospheres) are fed every 2-7 days, and more particularly every 2-4 days by gentle centrifugation and resuspension in medium containing a Wnt polypeptide or a growth factor.

After 6-7 days *in vitro*, individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, more particularly by titrating the neurospheres with a pipette. Single cells from the dissociated neurospheres are suspended in culture medium containing growth factors, and differentiation of the cells can be induced by plating (or resuspending) the cells in the presence of a Wnt agonist, and (optionally) any other factor capable of inducing and/or sustaining differentiation.

The tissue culture media is supplemented with a Wnt polypeptide (either by adding a Wnt polypeptide to

the culture media or by adding feeder cells producing a Wnt polypeptide) to maintain a stem cell phenotype of the precursor cells and to promote proliferation of the cells. Other commercially available growth factors such as Fibroblast Growth Factor (FGF) or Epidermal Growth Factor (EGF) are added to the culture as mitogens.

Cells cultured in the presence of a Wnt polypeptide, e.g., a member of the Wnt-1 class of polypeptides, proliferate and maintain a stem cell phenotype. Differentiation of the cells can proceed upon the removal of the Wnt polypeptide and/or addition of a composition that promotes differentiation.

A naturally-occurring population of neural crest cells contains multipotent (i.e., uncommitted) neural crest cells and committed precursor cells. The role of Wnt proteins employed in the present method is to culture a population of neural precursor cells, e.g., a naturally-occurring population of neural crest cells, (1) to induce cell fate of an uncommitted precursor and thereby give rise to a committed precursor cell and (2) to maintain such cells in a stem cell state (e.g., to arrest the development of a committed precursor cell towards becoming a terminally-differentiated neuronal cell). For example, the present method can be used *in vitro* to induce and/or maintain the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The Wnt protein can be used alone, or can be used in combination with other neurotrophic factors which act to more particularly enhance a particular differentiation fate of the neuronal precursor cell. In the later instance, an Wnt polypeptide might be viewed as ensuring that the treated cell has achieved a particular phenotypic state such that the cell is poised

along a certain developmental pathway so as to be properly induced upon contact with a secondary neurotrophic factor. Even relatively undifferentiated stem cells or primitive neuroblasts can be maintained in culture and caused to differentiate by treatment with Wnt agonists. Exemplary primitive cell cultures comprise cells harvested from the neural plate or neural tube of an embryo.

A population of neural precursor cells is characterized as having a stem cell phenotype when the level of proliferation of the cells in standard tissue culture media (e.g., MEM, DMEM, RPMI, F-12) in the presence of a Wnt polypeptide is at least 20% greater than the level of proliferation in the same tissue culture media without the Wnt polypeptide. Preferably, the level of cell proliferation is at least 50% greater in the presence of a Wnt polypeptide compared to the level of proliferation in the absence of a Wnt polypeptide. Proliferation is measured using known methods, e.g, incorporation of tritiated thymidine. Neural cells with a differentiated phenotype are characterized as non-proliferating and having the physical characteristics and cell markers of a mature terminally-differentiated neuron.

Primary stem cells may be immortalized by a variety of known techniques such as retrovirus-mediated transduction of an immortalizing gene, e.g., avian *myc* (*v-myc*).

<u>Graft preparation</u>

The therapeutic methods of the invention which utilize enriched populations of neural precursor cells may be used to treat neurodegenerative diseases and other types of diseases that result in depletion of neural cells. In addition to chronic depletion associated with progressive neurodegenerative diseases, neurons may be

killed as a consequence of infectious diseases, autoimmune diseases, and immunodeficiency diseases. Clinical outcome of treatment can be assessed by measuring as motor and cognitive capabilities of the patient, length of patient survival, quality of life.

Precursor cells cultured in the presence of a Wnt polypeptide as described above are washed and resusupended in a pharmaceutically acceptable excipient, e.g., a solution of 0.6% glucose-saline, are transplanted into brain tissue of a recipient mammal using known methods, e.g., those described by Gage et al., 1987, Ciba Found. Symp. 126:143-159. A small volume of a cell suspension is steriotaxically injected into a desired region, e.g., the hippocampus, of a mammal. For example, approximately 10^9 cells are infused into a desired location of the brain of the patient over 30 min.

Subsequent to transplantation, a Wnt polypeptide may be administered to the patient to induce further proliferation of stem cell *in vivo*. Wnt polypeptides can be administered in the form of a nerve prostheses for the repair of central and peripheral nerve damage. In particular, where a crushed or severed axon is intubulated by use of a prosthetic device, Wnt polypeptides can be added to the prosthetic device to increase the rate of growth and regeneration of the dendritic processes.

Alternatively, prior to transplantation, the cells may be exposed to a composition that induces differentiation <u>Treatment of neurodegenerative disease</u>

Neurodegenerative diseases include familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, Huntington's disease, familial and sporadic Alzheimer's disease, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse lewy

body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, gilles de la tourette syndrome, and Hallervorden-Spatz disease. Most of the diseases are typified by onset during the middle adult years and lead to rapid degeneration of specific subsets of neurons within the neural system, ultimately resulting in premature death. There is no known cure nor is there an effective therapy to slow the progression for any of the listed diseases.

Parkinson's disease (paralysis agitans) is a common neurodegenerative disorder which appears in mid to late life. Familial and sporadic cases occur, although familial cases account for only 1-2 percent of the observed cases. The neurological changes which cause this disease are somewhat variable and not fully understood. Patients frequently have nerve cell loss with reactive gliosis and Lewy bodies in the substantia nigra and locus coeruleus of the brain stem. Similar changes are observed in the nucleus basalis of Meynert. Nigrostriatal dopaminergic neurons are most affected.

The disorder generally develops asymmetrically with tremors in one hand or leg and progresses into symmetrical loss of voluntary movement. Eventually, the patient becomes incapacitated by rigidity and tremors. In the advanced stages the disease is frequently accompanied by dementia.

Diagnosis of both familial and sporadic cases of Parkinson's disease can only be made after the onset of the disease. Anticholinergic compounds, propranolol, primidone and levodopa are frequently administered to modify neural transmissions and thereby suppress the symptoms of the disease, though there is no known therapy which halts or slows the underlying progression. The therapeutic methods described herein may be administered

in conjunction with existing therapeutic approaches to neurodegenerative diseases.

The death of the dopaminergic neurons in the basal ganglia is an underlying defect of this progressive chronic disease as the basal ganglia are involved in the control of voluntary movements. Wnt-polypeptides and neural precursor cells cultured in the presence of Wnt polypeptides, e.g., Wnt-1, are useful in the treatment of Parkinson's disease and other disorders of midbrain dopamine circuitry. Transplantation of dopaminergic neural precursor cells is used to repopulate a patient's depleted population of dopaminergic neurons to treat or ameliorate the symptoms of Parkinson's disease.

Another neurodegenerative disease, Alzheimer's disease, can take two forms: disease exist: presenile dementia, in which the symptoms emerge during middle age, and senile dementia which occurs in the elderly. Both forms of the disease appear to have the same pathology. Diseases which affect learning and memory may be characterized by a depletion of hippocampal cells. Transplantation of hippocampal neural precursor cell is used to repopulate a patient's depleted population of hippocampal neurons to treat neurodegenerative diseases that affect learning and memory such as Alzheimer's disease.

Example 1: Wnt Signaling and Proliferation

Wnt signalling was found to regulate the expansion of dorsal neural precursors. Wnt-1 and Wnt-3a are coexpressed at the dorsal midline of the developing neural tube. Wnt-1 is involved in midbrain patterning, and Wnt-3a is involved in the formation of the paraxial mesoderm. The absence of a dorsal neural tube phenotype in animals with a mutation in either gene suggested that Wnt signalling is redundant. The data described below indicate that in the absence of both Wnt-1 and Wnt-3a,

there is a marked deficiency in neural crest derivatives, which originate from the dorsal neural tube, and a pronounced reduction in dorsolateral precursors within the neural tube itself.

Mice lacking both Wnt-1 and Wnt-3a signaling were generated. Mice which are heterozygous for null alleles of Wnt-1 and Wnt-3a were made using known methods (e.g., McMahon et al., 1990, Cell 62:1073-1085 and Takada et al., 1994, Genes Dev. 8:174-189). Compound heterozygotes (on a predominantly 129/Sv background) were intercrossed to recover compound mutants. Genotypes were confirmed by genomic Southern hybridization and polymerase chain reaction (PCR). Whole mount immunostaining was carried out using antibodies specific for neurofilaments, CRABP-1, and Lmx-1b. Skeletons from 18.5 d.p.c embryos were prepared and stained with alcian blue and alizarin red using known methods.

To evaluate cell proliferation and death, embryos were collected at 9.5 d.p.c (20-25 somite stage development) after intraperitoneal injection of pregnant females with 50 μg per body weight of 5-bromo-2'-deoxyuridine (BrdU). Mice were killed one hour later. Embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. After dehydration, wax embedding and sectioning at a thickness of 6 μm, serial sections were dewaxed and either stained with haematoxylin and eosin, or assayed for BrdU incorporation for apoptotic death using a standard TUNEL procedure.

Compound homozygotes were recovered at the expected Mendelian frequency (51 compound homozygotes in 673 embryos. The frequency was close to the expected frequency of 1/16) between 9.0 and 10.5 days post coitum (d.p.c.). Due to the termination of caudal axial development accompanying the loss of Wnt-3a activity,

relatively few of these embryos survived to 18.5 d.p.c. (3 compound homozygotes in 151 embryos).

To assess the development of the dorsal neural tube in compound mutants, neural crest derived structures were examined. Neural crest cells are among the first differentiated cell types to be formed by dorsal neural precursors. Neurofilament staining indicated that both neural crest derived cranial and spinal ganglia formation were unaltered in single mutants (either Wnt-1 or Wnt-3a mutants) which were either wild type or heterozygous for mutations in the other Wnt member. However, in double mutants, neurons derived from the proximal ganglion of cranial nerve IX (glossopharyngeal), which is formed by crest cells originating from rhombomere 6 within the hindbrain (r6), were absent. In contrast, the distal ganglion which is placodal in origin was present. In addition, there was a marked reduction in the proximal axons of cranial nerves V (trigeminal, r2 derived) and X (vagus, r7 derived). Similarly, in the trunk, there was a reduction in neurofilament staining in the cervical dorsal root ganglia. Further, cell counts indicated a 60% decrease in the cellular content of the dorsal root ganglia. Whole-mount *in situ* hybridization with probes specific for *Islet-1* and cadherin-6, markers of neuronal and glial neural crest derivatives, respectively, confirmed the reduction or absence of crest cells within the cranial ganglia and dorsal root ganglia. In contrast sympathetic ganglia, which express *c-ret*, were unaffected.

The reduction of neurogenic and gliogenic crest derivatives in the caudal head and rostral trunk regions indicates that fewer neural crest cells emerge in embryos lacking both Wnt-1 and Wnt-3a signaling. The issue of neural crest formation was evaluated by examining CRABP-1 immunoreactivtity and AP-2 transcription. CRABP-1 is

normally present in the dorsal CNS at 9.0 d.p.c. as well as in migrating neural crest cells arising from r2, 4 and 6. AP-2 is first expressed at 8.5 d.p.c. in the dorsal neural plate, coincident with neural crest formation. By 9.5 d.p.c. cranial expression is absent in the neural tube but persists in migrating and maturing neural crest derivatives at cranial and spinal cord levels. Loss of function studies have demonstrated that AP-2 is essential for development of neural crest derived structures. A clear decrease was observed in migrating CRABP-1 positive cells within the hindbrain, although CRABP-1 staining within the CNS appeared to be relatively normal. Similarly, examination of AP-2 expression revealed a reduction in both cranial and trunk neural crest. In contrast to their wild type litter mates, double mutants also retained AP-2 expression within the dorsal CNS at 9.5 d.p.c. Thus, in the absence of Wnt-1 and Wnt-3a, there is both a reduction in neural crest cell formation and persistent expression of AP-2 at the dorsal midline.

To determine whether Wnt-signaling was required throughout the period of cranial crest formation, expression of TRP-2 was evaluated. TRP-2 is a marker of presumptive melanocytes which are dominant in late formed cranial crest derivatives. At 11.5 d.p.c., TRP-2 expression was virtually absent within presumptive melanocytes migrating within the hindbrain region of double mutants though a few TRP-2 cells remained at the dorsal midline. In view of the prolonged expression of AP-2 within the dorsal CNS, TRP-2 expressing cells may be differentiating at a later stage, or they may be retained at the midline because Wnt-signaling promotes neural crest migration. Neither CRABP-1, TRP-2 or AP-2 expression was altered in the forebrain indicating that there is regional specificity in the requirement for these Wnt-signals.

Much of the head skeleton is generated by cranial neural crest. Distinct skeletal elements are derived from neural crest cells which emerge from different regions of the brain. To determine whether the reduction in neural crest formation in double mutants leads to alterations in the skeleton, 18.5 d.p.c. embryos were stained with alcian blue and alizarin red to examine cartilage and bone development. The stapes and the main body of the hyoid bone including the greater horn which originate from crest cells derived from r3-5 and r6-7, respectively, were absent. Thyroid cartilage showed a consistent dysmorphology. The reduction in hindbrain crest formation was also reflected in the absence of specific skeletal derivatives. In contrast, despite the early loss of forebrain, midbrain and rostral hindbrain in double mutants, the development of skeletal crest derivatives from these regions, as well as non-crest derived bones, was largely normal though there was some reduction in development of the squamosal, alisphenoid, basisphenoid, presphenoid and otic capsule. These data indicate that, in the absence of Wnt-1/3a signaling, several neural crest cell fates form, but there is a dramatic reduction in neural crest derivatives in the hindbrain region and in the spinal cord.

Neural crest cell development, and other aspects of dorsal polarity within the developing CNS, are thought to be regulated by BMP signals produced initially by the dorsal ectoderm and subsequently by the dorsal CNS. BMP-7 expression was induced, as expected, in the roof plate of double mutants. The data indicate that it was unlikely that defective neural crest development resulted from a secondary loss of BMP-signaling within the dorsal neural tube.

To determine whether Wnt-signaling directly regulates dorso-ventral polarity within the CNS, the

distribution of a number of regionally expressed markers was examined. Whereas spinal cord levels appeared normal, the hindbrain displayed a striking phenotype. Expression of Wnt-3a, Wnt-1 and Lmx-1b was normal in the roof plate. Thus, unlike other aspects of Wnt-signaling in the mammalian embryo, these Wnt-expressing cells did appear to require the Wnt-signals they produce. In contrast, expression of Math1 (which is activated at 9.5 d.p.c. in cells immediately adjacent to the roof plate) and Pax-3 (which occupies most of the dorsal half of the CNS) were dramatically reduced in the double mutant hindbrain. Dbx expression at the dorsal-ventral interface and Pax-6 expression in the ventro-lateral CNS were normal.

The data indicate that in the hindbrain, Wnt-signaling does not appear to play a role directly in the primary patterning processes which lead to the establishment of distinct cell fates in appropriate positions along the dorsoventral axis. Rather, it appears to play an essential role in the subsequent expansion of dorso-lateral neural progenitors. In support of a potential role in neural proliferation, transgenic analysis demonstrated that Wnt-1 can act as a potent mitogen when ectopically expressed within the dorsal CNS.

In normal development there is a ventral to dorsal progression in the formation of different neural crest derivatives. In the double mutants, the most severely affected crest derivatives were more proximal (dorsally located) structures. The stapes was absent from the second branchial arch while the lesser horn of the hyoid was unaltered, and in the trunk, dorsal root ganglia were markedly reduced while the sympathetic ganglia appeared normal. If the signals governing commitment to neural crest cell fates were unperturbed in the double mutant,

but renewal of a multipotential dorsal neural progenitor pool required Wnt-signals, the expected result would be a loss of later forming neural crest derivatives in Wnt-1/-3a mutants, as precursors within the neural tube became limiting.

Cell proliferation and cell death in hindbrain tissue sections (9.5 d.p.c; 20-25 somites) were analyzed using BrdU incorporation and TUNEL staining, respectively.
Dorsal neural precursors were reduced, but no discernible change was detected in either proliferation or cell death within remaining dorsal regions of Wnt-1 and Wnt-3a mutants. As these two Wnts are first coexpressed at the otic level when the first neural crest cells appear (at about 8.5 d.p.c; 8-10 somites), it is likely that the main reduction in dorsolateral neural precursors occurs between 8.5 and 9.5 d.p.c.

These data indicate that Wnt signalling regulates dorsoventral patterning in the mammalian CNS through the control of cell proliferation.

Example 2: Wnt-3A Signaling in Neuronal Differentiation

Wnt-3a expression in the mouse begins in the primitive streak region of the late egg cylinder at 7.5 d.p.c. and is maintained in the tail bud until tail formation is complete. To determine which cell types in the primitive streak region express Wnt-3a, the expression of Wnt-3a transcripts was examined in wild type embryos at the 7 somite stage. Expression was detected in the ectoderm layer in the primitive streak region but was absent from the node. Expression was further restricted for ventrally located cells in the anterior streak region. In contrast, in the posterior streak, most cells in the ectoderm layer expressed Wnt-3a. Wnt-3a expression was not observed in migrating mesodermal cells at either anterior or posterior

positions. These data indicate that Wnt-3a expression is localized to the primitive ectoderm prior to the physical segregation of the paraxial mesoderm and is downregulated as cells ingress through the primitive streak.

The phenotype of Wnt-3a homozygous mutant embryos was analyzed at early somite stages. At the 5 somite stage, no obvious differences in morphology between wild type and Wnt-3a mutant embryos were detected. However, by the 7 somite stage, differences in the shape of the primitive streak region were apparent. In Wnt-3a mutants, the width of the primitive streak region is narrower than in the wild type embryos and this phenotype becomes more pronounced by the 10 somite stage.

To further investigate the abnormal morphology of mutant embryo, histological analysis of the sections was carried out. In wild type embryos at the 7 somite stage, migrating presomitic mesodermal cells were observed under the primitive ectoderm layer in the primitive streak region. However, in Wnt-3a mutant embryos at the same stage, no migrating presomitic mesoderm cells were observed; in contrast, the shape and movement of cells ingressed through the primitive streak are quite different from those in normal embryos. In the anterior streak region of the mutant embryos, the ingressing cells were round in appearance, quite distinct from the usual stellate mesenchymal morphology of the ingressing mesoderm. Furthermore, these cells contacted each other and formed an ectopic tubular structure under the primitive streak at more posterior level. This tubular structure was not observed anterior to the streak where somites are present. Thus, in Wnt-3a mutant embryos, the absence of somite precursors appears to be correlated with the appearance of an ectopic tubular structure arising in the primitive streak region.

To identify the molecular characteristics of the ectopic tubular structure in Wnt-3a mutant embryos, *in situ* hybridization and whole mount immunostaining and the expression of a variety of molecular markers detected.

MF-1, encodes a forkhead domain containing protein, which is normally expressed in somites, presomitic mesoderm, and lateral mesoderm at 9.5 d.p.c. In Wnt-3a mutant embryos at this stage, no obvious MF-I expression was observed in the position where the ectopic tube was formed posterior to the forelimb level. A transverse section of the stained embryo at this axial level clearly indicated that no MF-1 transcripts were localized in the ectopic tube. Similarly another paraxial mesoderm marker, Mox-1, was not expressed in the ectopic tube in Wnt-3a mutants at either 8.5 or 9.5 d.p.c. The data indicate that the ectopic tube does not have the molecular and morphological characteristics of paraxial mesoderm.

Mash-I is normally expressed in central nervous system and peripheral nervous system precursors at 9.5 d.p.c. but not in the mesoderm. In Wnt-3a mutant embryos at the same stage, *Mash-1* expression was detected not only in these region but also in the region ventral to the original neural tube posterior to the forelimb level. A transverse section of Wnt-3a mutants at the axial level, where abnormal Mash-7 expression was observed, indicated that the ventral expression of Mash-I was localized in the ectopic tube. A second neural marker, HES-5, which is normally expressed in CNS, was also expressed in the ectopic tube in Wnt-3a mutants at 9.5 d.p.c. To explore further whether neurons differentiate in the ectopic tube, Wnt-3a mutant embryos at 10.5 d.p.c. were immunostained with antineurofilament antibody, 2H3. Neurofilament expressing cells were present in both the dorsal neural tube and the ectopic ventral tube.

The ectopic tube also exhibited polarity typical of CNS tissue. For example, Sonic hedgehog (Shh) is normally expressed in the floor plate of the neural tube. In 9.5 d.p.c. Wnt-3a mutant embryos, the notochord was present under the ventral ectopic tubular structure but not under the original neural tube at the axial level just posterior to the forelimbs while no notochord was absorbed at more posterior levels. Shh was expressed in ventrally in the ectopic tube where it contacts the notochord, suggesting, that the ectopic tube forms a floor plate in response to a Shh signaling by the notochord. The ectopic neural tube also exhibits dorsal polarity typical of the CNS such as the expression of the dorsal midline marker, Wnt-1 and increased levels of Pax-3 expression, where the tube contacts the surface ectoderm. In addition, expression of a ventral CNS marker, Pax-6, was suppressed where the ectopic tube contacts the surface ectoderm. Taken together, the data indicate that the ectopic tubular structure in the mutants has the molecular and cellular characteristics of an ectopic neural tube and consequently the loss of Wnt-3a signaling results in the formation of CNS precursors at the expense of paraxial mesoderm.

The phenotype of Wnt-3a knock out mutant embryos at 9.5 d.p.c. indicated that Wnt-3a is essential for formation of somitic mesoderm caudal to first 7-9 somites. In the absence of somite formation, an ectopic tubular structure which displays both cellular and molecular characteristics of presumptive CNS tissue is formed. Several lines of evidences suggest that the neural tube was formed ectopically. First, transverse sections of Wnt-3a mutant embryos at an early somite stage indicated that cells delaminating from and ingressing through the primitive streak form an epithelial cell layer that contribute to an ectopic tube

under the primitive ectoderm in the primitive streak region. Second, the notochord contacts the ventral but not the dorsal neural tube, suggesting that the ventral (ectopic) neural tube had already formed at the time when the notochord was laid down. Third, by the analysis of serial transverse sections of several 8.5 and 9.5 d.p.c. Wnt-3a mutant embryos, it is apparent that the ectopic neural tube is not continuous with the original dorsal neural tube suggesting an independent origin.

The appearance of the ectopic neural tube correlates with the disappearance of the paraxial mesoderm precursors in Wnt-3a mutant embryos. This correlation suggests that the absence of Wnt-3a signaling in the primitive ectoderm of the primitive streak may lead to presumptive somitic mesoderm cells to adopting, neural cell fate. That is, a neural fate may be a "default" state for cells which normally give rise to both mesodermal and neural derivatives.

The results described herein indicate that in the normal primitive ectoderm, where Wnt-3a is expressed, undifferentiated cells can differentiate into both neural and somitic mesoderm cell lineages. At early somite stages, cells in the anterior primitive streak generate mostly somitic mesoderm, while cells in the posterior streak gives rise to mostly lateral mesoderm. In contrast, primitive ectoderm adjacent to the anterior primitive streak contributes mainly to somitic mesoderm and neuroectoderm, suggesting that these two cell types might arise from the same cell population. The data indicate that Wnt-3a signaling regulates cell fate specification between somitic mesoderm and neural lineages in the normal mouse embryo.

Although Wnt-3a is expressed in the anterior streak in regions which gives rise to somitic mesoderm, it is also expressed in more posterior regions which

generate lateral and ventral mesoderm. Thus, expression is not restricted to paraxial mesoderm precursors. Wnt-3a may establish a competence to respond to a paraxial mesoderm inducing signal, rather than itself directly inducing paraxial mesodermal cell fates. This competence may be broadly distributed within the streak.

Example 3: Wnt-1 signaling and mid-brain development

Expression of En-1 in the developing midbrain of Wnt-1 null embryos is sufficient to rescue midbrain and interior hindbrain development. In the mouse, Wnt-1 and Engrailed-1 (En-1) are first expressed in the presumptive midbrain, from 8.0 days post coitum (d.p.c.) and continue to be expressed, together with En-2, in overlapping patterns during midbrain development. In Wnt-$1^{-/-}$ (Wnt-1-null) embryos, En-1 and En-2 expression is initiated normally, but subsequently both domains of En expression are lost, which is concomitant with a failure of midbrain and anterior hindbrain development.

En-1 was expressed from the transgene WEXPZ-En-1 in a pattern similar to that of endogenous Wnt-1 gene. To assess whether En-1 was able to rescue the Wnt-1-null phenotype, embryos from matings of Wnt-$1^{+/-}$, WEXPZ-En-1^{+} males with Wnt-$1^{+/-}$ females were collected at 14.5 d.p.c., when the Wnt-$1^{-/-}$ phenotype can easily be scored morphologically. The genotype was subsequently determined by southern blotting. Wnt-$1^{+/+}$ and Wnt-$1^{+/-}$ embryos with or without WEXPZ-En-1 appeared to be wild-type (n = 112) whereas all Wnt-$1^{-/-}$ embryos (n = 12) displayed the Wnt-$1^{-/-}$ phenotype. In Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos, 7 out of 17 appeared superficially wildtype, 8 out of 17 were partially rescued and only 2 out of 17 were similar to Wnt-$1^{-/-}$ embryos.

To characterize brain development in greater detail, a minimum of four embryos from each category were

sectioned for histological analysis. All Wnt-$1^{-/-}$ embryos lacked the midbrain and cerebellum. In contrast, in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos that were scored as wild-type, the midbrain and cerebellum appeared similar to those of wild-type embryos. In partially rescued embryos, only the posterior midbrain and a slightly reduced cerebellum were apparent. The absence of rescue in some cases, and partial rescue in others, may reflect influences of the genetic background or variations in the levels of En-1 expressed from the transgene.

To characterize the development of the midbrain in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos further, the expression of several genes normally transcribed in this region was examined at 10.5 d.p.c. Pax-5 is expressed in a broad domain at the midbrain-hindbrain junction, but this domain is missing in Wnt-$1^{-/-}$ embryos. In Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos, Pax-5 expression was detected in a pattern similar to that of the wild-type embryos. Wnt-1 and Fgf -8 are normally expressed in adjacent rings of cells just anterior and posterior to the midbrain-hindbrain junction, respectively. Fgf8 signalling is involved in midbrain development. In Wnt-$1^{-/-}$ embryos, both rings of expressing cells are absent. In contrast, both Wnt-1 and Fgf-8 were expressed in sharp rings of cells in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos despite the fact that no morphologically obvious midbrain-hindbrain junction was apparent. These results indicate that Wnt-1 signaling at this later stage may not play a direct role in regulating Fgf-8 expression in adjacent cells. En gene expression was also restored in the mid-hindbrain region of Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos outside the area where the transgene is expressed.

In all the rescued embryos, the expression domains of Pax-5, Fgf-8, En, and, in a few cases, Wnt-1 were

slightly reduced relative to wild-type littermates (18 out 41 Wnt-$1^{-/}$, WEXPZ-En-1^{+} embryos expressed one of the markers examined, of these at least half were substantially rescued). One likely explanation is that rescued embryos have a smaller population of midbrain cells than wild-type siblings because when Wnt-1 and En-1 expression is initiated, Wnt-1 mRNA transcription is patchy, whereas En genes are expressed more uniformly in presumptive midbrain cells. Thus, in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos, where En-1 is not uniformly expressed in all presumptive midbrain cells, only those cells that express En-1 at this early stage may contribute to midbrain development. As En-1 expression in the midbrain restores Fgf-8, Pax-5 and En expression in the anterior hindbrain, and subsequently cerebellum development in Wnt-$1^{-/-}$ embryos, the data suggest that a midbrain-derived signal other than Wnt-1 is necessary for anterior hindbrain development.

To assess whether expression of En-1 was sufficient to rescue the viability of Wnt-$1^{-/-}$ mice (pups are born but die within 24 h) pups were genotyped at 10 days post partum (n = 68). No live Wnt-$1^{-/-}$, WEXPZ-En-1^{+} mice were obtained indicating that En-1 was insufficient to rescue the Wnt-1-null phenotype completely. Further analysis indicated that between 14.5 and 18.5 d.p.c., brains of Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos deteriorate, indicating that there may be additional functions of Wnt-1 signaling that cannot be replaced by En-1. This conclusion is supported by analysis of two cranial motor nerves, III (oculomotor) and IV (trochlear), which normally develop adjacent to Wnt-1-expressing cells in the ventral midbrain. Each of these fail to develop in Wnt-$1^{-/-}$ embryos. Similarly, neither nerve forms in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos which have

global restoration of midbrain development. In contrast, a second ventral population, tyrosine-hydroxylase-expressing neurons (catecholaminergic neurons) of the substantia nigra, are rescued in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos.

These data demonstrate that, in the absence of a Wnt-1 signal, expression of En-1 from the Wnt-1 enhancer is sufficient to substantially rescue early midbrain and anterior hindbrain development, and suggest that a major role of Wnt-1 signalling in the mammalian brain is to maintain En expression.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: President and Fellows of Harvard College

(ii) TITLE OF INVENTION: INDUCTION OF NEURONAL REGENERATION

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US98/-----
(B) FILING DATE: 30-APR-1998

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Freeman, John W.
(B) REGISTRATION NUMBER: 29,066
(C) REFERENCE/DOCKET NUMBER: 00246/222WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/542-5070
(B) TELEFAX: 617/542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 370 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Gly Leu Trp Ala Leu Leu Pro Gly Trp Val Ser Ala Thr Leu Leu
 1               5                  10                  15
Leu Ala Leu Ala Ala Leu Pro Ala Ala Leu Ala Ala Asn Ser Ser Gly
            20                  25                  30
Arg Trp Trp Gly Ile Val Asn Val Ala Ser Ser Thr Asn Leu Leu Thr
        35                  40                  45
Asp Ser Lys Ser Leu Gln Leu Val Leu Glu Pro Ser Leu Gln Leu Leu
    50                  55                  60
Ser Arg Lys Gln Arg Arg Leu Ile Arg Gln Asn Pro Gly Ile Leu His
```

```
65                  70                      75                  80
Ser Val Ser Gly Gly Leu Gln Ser Ala Val Arg Glu Cys Lys Trp Gln
                85                      90                  95
Phe Arg Asn Arg Arg Trp Asn Cys Pro Thr Ala Pro Gly Pro His Leu
            100                 105                 110
Phe Gly Lys Ile Val Asn Arg Gly Cys Arg Glu Thr Ala Phe Ile Phe
        115                 120                 125
Ala Ile Thr Ser Ala Gly Val Thr His Ser Val Ala Arg Ser Cys Ser
    130                 135                 140
Glu Gly Ser Ile Glu Ser Cys Thr Cys Asp Tyr Arg Arg Arg Gly Pro
145                 150                 155                 160
Gly Gly Pro Asp Trp His Trp Gly Gly Cys Ser Asp Asn Ile Asp Phe
                165                 170                 175
Gly Arg Leu Phe Gly Arg Glu Phe Val Asp Ser Gly Glu Lys Gly Arg
            180                 185                 190
Asp Leu Arg Phe Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Thr
        195                 200                 205
Thr Val Phe Ser Glu Met Arg Gln Glu Cys Lys Cys His Gly Met Ser
    210                 215                 220
Gly Ser Cys Thr Val Arg Thr Cys Trp Met Arg Leu Pro Thr Leu Arg
225                 230                 235                 240
Ala Val Gly Asp Val Leu Arg Asp Arg Phe Asp Gly Ala Ser Arg Val
                245                 250                 255
Leu Tyr Gly Asn Arg Gly Ser Asn Arg Ala Ser Arg Ala Glu Leu Leu
            260                 265                 270
Arg Leu Glu Pro Glu Asp Pro Ala His Lys Pro Pro Ser Pro His Asp
        275                 280                 285
Leu Val Tyr Phe Glu Lys Ser Pro Asn Phe Cys Thr Tyr Ser Gly Arg
    290                 295                 300
Leu Gly Thr Ala Gly Thr Ala Gly Arg Ala Cys Asn Ser Ser Ser Pro
305                 310                 315                 320
Ala Leu Asp Gly Cys Glu Leu Leu Cys Cys Gly Arg Gly His Arg Thr
                325                 330                 335
Arg Thr Gln Arg Val Thr Glu Arg Cys Asn Cys Thr Phe His Trp Cys
            340                 345                 350
Cys His Val Ser Cys Arg Asn Cys Thr His Thr Arg Val Leu His Glu
        355                 360                 365
Cys Leu
    370
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Ala Pro Leu Gly Gly Ile Trp Leu Trp Leu Pro Leu Leu Leu
 1               5                  10                  15
Thr Trp Leu Thr Pro Glu Val Asn Ser Ser Trp Trp Tyr Met Arg Ala
            20                  25                  30
Thr Gly Gly Ser Ser Arg Val Met Cys Asp Asn Val Pro Gly Leu Val
        35                  40                  45
Ser Ser Gln Arg Gln Leu Cys His Arg His Pro Asp Val Met Arg Ala
    50                  55                  60
Ile Ser Gln Gly Val Ala Glu Trp Thr Ala Glu Cys Gln His Gln Phe
65                  70                  75                  80
Arg Gln His Arg Trp Asn Cys Asn Thr Leu Asp Arg Asp His Ser Leu
```

```
                    85                      90                      95
Phe Gly Arg Val Leu Leu Arg Ser Ser Arg Glu Ser Ala Phe Val Tyr
                100                 105                 110
Ala Ile Ser Ser Ala Gly Val Val Phe Ala Ile Thr Arg Ala Cys Ser
            115                 120                 125
Gln Gly Glu Val Lys Ser Cys Ser Cys Asp Pro Lys Lys Met Gly Ser
    130                 135                 140
Ala Lys Asp Ser Lys Gly Ile Phe Asp Trp Gly Gly Cys Ser Asp Asn
145                 150                 155                     160
Ile Asp Tyr Gly Ile Lys Phe Ala Arg Ala Phe Val Asp Ala Lys Glu
                165                     170                 175
Arg Lys Gly Lys Asp Ala Arg Ala Leu Met Asn Leu His Asn Asn Arg
            180                 185                 190
Ala Gly Arg Lys Ala Val Lys Arg Phe Leu Lys Gln Glu Cys Lys Cys
        195                 200                 205
His Gly Val Ser Gly Ser Cys Thr Leu Arg Thr Cys Trp Leu Ala Met
    210                 215                 220
Ala Asp Phe Arg Lys Thr Gly Asp Tyr Leu Trp Arg Lys Tyr Asn Gly
225                 230                 235                     240
Ala Ile Gln Val Val Met Asn Gln Asp Gly Thr Gly Phe Thr Val Ala
                245                 250                 255
Asn Glu Arg Phe Lys Lys Pro Thr Lys Asn Asp Leu Val Tyr Phe Glu
            260                 265                 270
Asn Ser Pro Asp Tyr Cys Ile Arg Asp Arg Glu Ala Gly Ser Leu Gly
        275                 280                 285
Thr Ala Gly Arg Val Cys Asn Leu Thr Ser Arg Gly Met Asp Ser Cys
    290                 295                 300
Glu Val Met Cys Cys Gly Arg Gly Tyr Asp Thr Ser His Val Thr Arg
305                 310                 315                     320
Met Thr Lys Cys Gly Cys Lys Phe His Trp Cys Cys Ala Val Arg Cys
                325                 330                 335
Gln Asp Cys Leu Glu Ala Leu Asp Val His Thr Cys Lys Ala Pro Lys
            340                 345                 350
Asn Ala Asp Trp Thr Thr Ala Thr
        355                 360
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 352 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Ala Pro Leu Gly Tyr Leu Leu Val Leu Cys Ser Leu Lys Gln Ala
1               5                   10                  15
Leu Gly Ser Tyr Pro Ile Trp Trp Ser Leu Ala Val Gly Pro Gln Tyr
            20                  25                  30
Ser Ser Leu Ser Thr Gln Pro Ile Leu Cys Ala Ser Ile Pro Gly Leu
        35                  40                  45
Val Pro Lys Gln Leu Arg Phe Cys Arg Asn Tyr Val Glu Ile Met Pro
    50                  55                  60
Ser Val Ala Glu Gly Val Lys Ala Gly Ile Gln Glu Cys Gln His Gln
65                  70                  75                      80
Phe Arg Gly Arg Arg Trp Asn Cys Thr Thr Val Ser Asn Ser Leu Ala
                85                  90                  95
Ile Phe Gly Pro Val Leu Asp Lys Ala Thr Arg Glu Ser Ala Phe Val
            100                 105                 110
His Ala Ile Ala Ser Ala Gly Val Ala Phe Ala Val Thr Arg Ser Cys
```

```
            115                     120                     125
Ala Glu Gly Ser Ala Ala Ile Cys Gly Cys Ser Ser Arg Leu Gln Gly
        130                 135                     140
Ser Pro Gly Glu Gly Trp Lys Trp Gly Gly Cys Ser Glu Asp Ile Glu
145                 150                     155                 160
Phe Gly Gly Met Val Ser Arg Glu Phe Ala Asp Ala Arg Glu Asn Arg
                165                     170                 175
Pro Asp Ala Arg Ser Ala Met Asn Arg His Asn Asn Glu Ala Gly Arg
            180                     185                 190
Gln Ala Ile Ala Ser His Met His Leu Lys Cys Lys Cys His Gly Leu
        195                     200                 205
Ser Gly Ser Cys Glu Val Lys Thr Cys Trp Trp Ser Gln Pro Asp Phe
    210                     215                 220
Arg Thr Ile Gly Asp Phe Leu Lys Asp Lys Tyr Asp Ser Ala Ser Glu
225                 230                     235                 240
Met Val Val Glu Lys His Arg Glu Ser Arg Gly Trp Val Glu Thr Leu
                245                     250                 255
Arg Pro Arg Tyr Thr Tyr Phe Lys Val Pro Thr Glu Arg Asp Leu Val
            260                     265                 270
Tyr Tyr Glu Ala Ser Pro Asn Phe Cys Glu Pro Asn Pro Glu Thr Gly
        275                     280                 285
Ser Phe Gly Thr Arg Asp Arg Thr Cys Asn Val Ser Ser His Gly Ile
    290                     295                 300
Asp Gly Cys Asp Leu Leu Cys Cys Gly Arg Gly His Asn Ala Arg Thr
305                 310                     315                 320
Glu Arg Arg Arg Glu Lys Cys His Cys Val Phe His Trp Cys Cys Tyr
                325                     330                 335
Val Ser Cys Gln Glu Cys Thr Arg Val Tyr Asp Val His Thr Cys Lys
            340                     345                 350
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 349 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Asn Arg Lys Ala Leu Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1               5                   10                  15
Gly Met Val Cys Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
            20                  25                  30
Gly Ala Thr Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
        35                  40                  45
Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
    50                  55                  60
Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65                  70                  75                  80
Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
                85                  90                  95
Leu Lys Val Gly Ser Arg Asp Gly Ala Phe Thr Tyr Ala Ile Ile Ala
            100                 105                 110
Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr His Gly Asn Leu
        115                 120                 125
Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
    130                 135                 140
Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145                 150                 155                 160
Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
                165                 170                 175
```

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190
Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205
Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220
Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240
Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
245 250 255
Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270
Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285
Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300
Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320
Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335
Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
340 345

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Val Ser Gly Ser Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro
1 5 10 15
Lys Phe Arg Glu Val Gly His Leu Leu Lys Glu Lys Tyr Asn Ala Ala
20 25 30
Val Gln Val Glu Val Val Arg Ala Ser Arg Leu Arg Gln Pro Thr Phe
35 40 45
Leu Arg Ile Lys Gln Leu Arg Ser Tyr Gln Lys Pro Met Glu Thr Asp
50 55 60
Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Ala Ala
65 70 75 80
Thr Gly Ser Val Gly Thr Gln Gly Arg Ile Cys Asn Arg Thr Ser Pro
85 90 95
Gly Ala Asp Gly Cys Asp Thr Met Cys Cys Gly Arg Gly Tyr Asn Thr
100 105 110
His Gln Tyr Thr Lys Val Trp Gln Cys Asn Cys Lys
115 120

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 365 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Gly Ser Ala Met Ser Ser Lys Phe Phe Leu Val Ala Leu Ala

```
1               5                  10                  15
Ile Phe Phe Ser Phe Ala Gln Val Val Ile Glu Ala Asn Ser Trp Trp
            20                  25                  30
Ser Leu Gly Met Asn Asn Pro Val Gln Met Ser Glu Val Tyr Ile Ile
        35                  40                  45
Gly Ala Gln Pro Leu Cys Ser Gln Leu Ala Gly Leu Ser Gln Gly Gln
    50                  55                  60
Lys Lys Leu Cys His Leu Tyr Gln Asp His Met Gln Tyr Ile Gly Glu
65                  70                  75                  80
Gly Ala Lys Thr Gly Ile Lys Glu Cys Gln Tyr Gln Phe Arg His Arg
                85                  90                  95
Arg Trp Asn Cys Ser Thr Val Asp Asn Thr Ser Val Phe Gly Arg Val
            100                 105                 110
Met Gln Ile Gly Ser Arg Glu Thr Ala Phe Thr Tyr Ala Val Ser Ala
        115                 120                 125
Ala Gly Val Val Asn Ala Met Ser Arg Ala Cys Arg Glu Gly Glu Leu
    130                 135                 140
Ser Thr Cys Gly Cys Ser Arg Ala Ala Arg Pro Lys Asp Leu Pro Arg
145                 150                 155                 160
Asp Trp Leu Trp Gly Gly Cys Gly Asp Asn Ile Asp Tyr Gly Tyr Arg
                165                 170                 175
Phe Ala Lys Glu Phe Val Asp Ala Arg Glu Arg Glu Arg Ile His Ala
            180                 185                 190
Lys Gly Ser Tyr Glu Ser Ala Arg Ile Leu Met Asn Leu His Asn Asn
        195                 200                 205
Glu Ala Gly Arg Arg Thr Val Tyr Asn Leu Ala Asp Val Ala Cys Lys
    210                 215                 220
Cys His Gly Val Ser Gly Ser Cys Ser Leu Lys Thr Cys Trp Leu Gln
225                 230                 235                 240
Leu Ala Asp Phe Arg Lys Val Gly Asp Ala Leu Lys Glu Lys Tyr Asp
                245                 250                 255
Ser Ala Ala Ala Met Arg Leu Asn Ser Arg Gly Lys Leu Val Gln Val
            260                 265                 270
Asn Ser Arg Phe Asn Ser Pro Thr Thr Gln Asp Leu Val Tyr Ile Asp
        275                 280                 285
Pro Ser Pro Asp Tyr Cys Val Arg Asn Glu Ser Thr Gly Ser Leu Gly
    290                 295                 300
Thr Gln Gly Arg Leu Cys Asn Lys Thr Ser Glu Gly Met Asp Gly Cys
305                 310                 315                 320
Glu Leu Met Cys Cys Gly Arg Gly Tyr Asp Gln Phe Lys Thr Val Gln
                325                 330                 335
Thr Glu Arg Cys His Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
            340                 345                 350
Lys Lys Cys Thr Glu Ile Val Asp Gln Phe Val Cys Lys
        355                 360                 365
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5607 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGTATGTAT GTATGTATGT ATGTATGTAT ACGTGCGTGC ACCTGTGTGT GCTTGGTGTC
60
AGTGGGGCTC AGACATCACC TGATTCCCTG GAACTGGAGT TACAGGTGGC TATAAGCCAC
120
```

CACTTGGGTG CTGAGAACAG AGTCCGGGCC TCTGGCAGAG CAGTCAGTGC TTTTAGCCAC
180
TGAGCCACTC TCATCCCCCC AATTATGTTC ATCTTGAGTT GGGCAGGTAC GGTGGCGGAA
240
TAGGCCTGTA ATCCCAGCAG TCACTGGACC ATCATGGGTT CTACATATTA AACCTTTATG
300
TTAGGTAGGG TCACACAGCA AGATCCGGTC ACAAAACCAG CAACAACAAA AACCAAAAGG
360
AGCCAGCTTC TTCCCACAAG CATTCTTTCC CTCAGGTCTT CAGCTCCATC TGACAGCTAC
420
TCGGCTGGTG GTCCTATCCT TTCTGAGCCT AGTTGCCAGA GAAACAAGCC CGGTTCATCT
480
TCATGACTAG CACATCTAAT GATAAGCACA GGTTGACTCA AGGTGCCATA GAGTGACACT
540
AGGTACCCAG AGCGACAGAA TGACACCTAT GAGTGCACGT CGTTAATCAC AAACACACAC
600
ACACACACAC ACACACACAC ACACACACAC TCATGCACCC ACCTGCAAAC ACAATTGCAG
660
CCTTCTGGAC GTCTCCTGTC ACAGCCCCAC CTCCTTCCTG ATACACTGCG TTAAGTGGTG
720
ACTGTAACAA AATGACTTCA TGCTCTCCCT GTCCTGAGCC AAATTACACA ATTATTTGGA
780
AAGGGCTCAA AATGTTCTTC GTTAGAAGTT TCTGGATACA CCAATACACA GGAGCGTGCA
840
CCCTCAGAAC ACATGTACAC TTTGACTTAA TCTCACGGGT GACACACCGA CGCTTACACT
900
CCCCCTAGCC CACAGAGGCA AACTGCTGGG CGCTTCTGAG TTTCTCACTG CCACCAGCTC
960
GGTTTGCTCA GCCTACCCCC GCACCCCGCG CCCGGGAATC CCTGACCACA GCTCCACCCA
1020
TGCTCTGTCT CCTTCTTTTC CTTCTCTGTC CAGCCGTCGG GGTTCCTGGG TGAGGAAGTG
1080
TCTCCACGGA GTCGCTGGCT AGAACCACAA CTTTCATCCT GCCATTCAGA ATAGGGAAGA
1140
GAAGAGACCA CAGCGTAGGG GGGACAGAGG AGACGGACTT CGAGAGGACA GCCCCACCGG
1200
CGCGTGTGGG GGAGGCAATC CAGGCTGCAA ACAGGTTGTC CCCAGCGCAT TGTCCCCGCG
1260
CCCCCTGGCG GATGCTGGTC CCCGACGGGC TCCGGACGCG CAGAAGAGTG AGGCCGGCGC
1320
GCGTGGGAGG CCATCCCAAG GGGAGGGGTC GGCGGCCAGT GCAGACCTGG AGGCGGGGCC
1380
ACCAGGCAGG GGGCGGGGGT GAGCCCCGAC GGTTAGCCTG TCAGCTCTTT GCTCAGACCG
1440
GCAAGAGCCA CAGCTTCGCT CGCCACTCAT TGTCTGTGGC CCTGACCAGT GCGCCCTGGT
1500
GCTTTTAGTG CCGCCCGGGC CCGGAGGGGC AGCCTCTTCT CACTGCAGTC AGCGCCGCAA
1560
CTATAAGAGG CCTATAAGAG GCGGTGCCTC CCGCAGTGGC TGCTTCAGCC CAGCAGCCAG
1620
GACAGCGAAC CATGCTGCCT GCGGCCCGCC TCCAGACTTA TTAGAGCCAG CCTGGGAACT
1680
CGCATCACTG CCCTCACCGC TGTGTCCAGT CCCACCGTCG CGGACAGCAA CCACAGTCGT
1740
CAGAACCGCA GCACAGAACC AGCAAGGCCA GGCAGGCCAT GGGGCTCTGG GCGCTGCTGC
1800
CCAGCTGGGT TTCTACTACG TTGCTACTGG CACTGACCGC TCTGCCCGCA GCCCTGGCTG
1860
CCAACAGTAG TGGCCGATGG TGGTAAGTGA GCTAGTACGG GGTCCGCCAC TTGTCCTGGG
1920
GCAAAGAGCC AGGCACGGGC CTTACCCAGC TCCCACGCTG TGGGGATCAC CAACCTACAG
1980

ACCCCCCTCG TGCATTGTGA CTTCACATCC AGGGTGCTCA CACCTAGAAC TAGCTCTGCT
2040
GAAGTGGGGC ACATCATTGG CATGCAGAAG CCCAGATACA CCAGGCTCAG AGACCATTCC
2100
CATTTAATAC GACCCCGTTT CTGCTGAGCA ACAGGTCCCA ACCTCGCTGT GGTGGGTGCT
2160
CAGGTGTCCC TTAGGTCTTG AACCAAAAAA AAAAAAAAAA AAAAAAAAAA ACCAGATATT
2220
AGCTTTGAGG TGAGGGAGTG GAATTCCTAA GTTTTTCAAG GTGGGCAAGG CTGCAGGTGG
2280
GGTTTCTCCT CGGGGGCTGA CTTGAAGAAA GGAAGAGCTA AGGTAGCCAT GCCTTTTCTG
2340
TCCACTCACT AGACTCTGGA GCTCAGGGCC AGGCAAGGAT AGGGTGGTAC AGCCTGTATG
2400
GTTAGGATGC AGGTCCCCTC CCCTGGACTG AACCCTTATG CATCCCGCCA GGGGCATCGT
2460
GAACATAGCC TCCTCCACGA ACCTGTTGAC GGATTCCAAG AGTCTGCAGC TGGTGCTCGA
2520
GCCCAGTCTG CAGCTGCTGA GCCGCAAGCA GCGGCGACTG ATCCGACAGA ACCCGGGGAT
2580
CCTGCACAGC GTGAGTGGAG GGCTCCAGAG CGCTGTGCGA GAGTGCAAAT GGCAATTCCG
2640
AAACCGCCGC TGGAACTGCC CCACTGCTCC GGGGCCCCAC CTCTTCGGCA AGATCGTCAA
2700
CCGAGGTGGG TGCCCAGGAA AGCGACGCTT CCGGGATTAA GGGAAAAGCA GGGTCATCTC
2760
CAGGGCATAG GCGGGCGAAG GCAGGGAAGA CATCCCAGGG TTATATGTGA TCAAACTGAG
2820
AATCGCCTGG TGCCGGCAGT TACCGTAGGT CAGCACCAGA TTCTTTCTAG CCTTGCGTTG
2880
TGAGCATGAT CTTTAACGTT GCTGGCCACT GGCCCACAGA AAGGGAATTC CGGATCGTGG
2940
GCGCTGGGCG ACAGCTGTTT TTCCCTAGCC TTCCTCAAAG GTACCTGGGA AGCTGATCTC
3000
TGAGGGCTAG CTAGGGTTGT GCTTCGCACC CAGCAAAGTT TGCACTGCCA ATACTAGTAG
3060
CGATCTTGGC TATGCAGATT TGTTCTACTT GGGAATCTCC CCTTGGAGCT GCTCTGCTAG
3120
GGCTCTGGAG TCTCAGTAAA GCTTAGAGAG GAGGGCATTC CATGCTTCGC ACACATGACT
3180
CCAAGGATGT TGGACTGTAG GGTACCAAGT CTTCCAAACA GGGTGCTGAG TTGGCCCCAC
3240
GCCTTCTCTC AACTGATGCG GGGTCGCTTC ACCCACAGGC TGCCGAGAAA CAGCGTTCAT
3300
CTTCGCAATC ACCTCCGCCG GGGTCACACA TTCCGTGGCG CGCTCCTGCT CCGAAGGCTC
3360
CATCGAGTCC TGCACCTGCG ACTACCGGCG GCGCGGCCCT GGGGGCCCCG ACTGGCACTG
3420
GGGGGGCTGC AGTGACAACA TCGATTTTGG TCGCCTCTTT GGCCGAGAGT TCGTGGACTC
3480
CGGGGAGAAG GGGCGGGACC TACGCTTCCT CATGAACCTT CACAACAACG AGGCAGGGCG
3540
AACGGTACGT CGGTGTGTCC GGAACCAATG GCAGGGGAGA TGTAAGACAG GTGCACGGGG
3600
ACAGAGGCAC AGGGAGGGGC TTCCCGAGAG AGTGGGACTC TAGGAGGGAA GACAGAGAAG
3660
AGGTGGTGGT TGAGGGCAAA GAGGTTCCTG AGCTGATGAC AGAACAGAAG AGATTAGCAG
3720
GCTATCAACA CGTGGGATGT ATTGAGATGG CTCCATGGCA CACTTTTGAA AGATAAAAGT
3780
GACTTGCTGG CGTGGAGCAG AGTCTGGCCG AATGTCCCTA TCTCAGCGGG CCATTTTGCA
3840

CTTCCTCTCT CCCGAGCTTA GTCACACCTG GACCTTGGCT GAAGTTTCCA CAGCATCGAC
3900
GTGACCCGGG TGGGGTGGGG GTGGGGAAGT ATGGGTGGTG GTTCGTGGGA TGTTGGCTTT
3960
GACCTTTTCT TCCCTCCTCC CCTCGTCCCC TCCTCCCCCA GACCGTGTTC TCTGAGATGC
4020
GCCAAGAGTG CAAATGCCAC GGGATGTCCG GCTCCTGCAC GGTGCGCACG TGTTGGATGC
4080
GGCTGCCCAC GCTGCGCGCT GTGGGCGACG TGCTGCGCGA CCGCTTCGAC GGCGCCTCCC
4140
GCGTCCTTTA CGGCAACCGA GGCAGCAACC GCGCCTCGCG GGCGGAGCTG CTGCGCCTGG
4200
AGCCCGAAGA CCCCGCGCAC AAGCCTCCCT CCCCTCACGA CCTCGTCTAC TTCGAGAAAT
4260
CGCCCAACTT CTGCACGTAC AGTGGCCGCC TGGGCACAGC TGGCACAGCT GGACGAGCTT
4320
GCAACAGCTC GTCTCCCGCG CTGGACGGCT GTGAGCTGCT GTGCTGTGGC CGAGGCCACC
4380
GCACGCGCAC GCAGCGCGTC ACGGAGCGCT GCAACTGCAC CTTCCACTGG TGCTGCCACG
4440
TCAGCTGCCG CAACTGCACG CACACGCGCG TTCTGCACGA GTGTCTATGA GGTGCCGCGC
4500
CTCCGGGAAC GGGAACGCTC TCTTCCAGTT CTCAGACACA CTCGCTGGTC CTGATGTTTG
4560
CCCACCCTAC CGCGTCCAGC CACAGTCCCA GGGTTCATAG CGATCCATCT CTCCCACCTC
4620
CTACCTGGGG ACTCCTGAAA CCACTTGCCT GAGTCGGCTC GAACCCTTTT GCCATCCTGA
4680
GGGCCCTGAC CCAGCCTACC TCCCTCCCTC TTTGAGGGAG ACTCCTTTTG CACTGCCCCC
4740
CAATTTGGCC AGAGGGTGAG AGAAAGATTC TTCTTCTGGG GTGGGGGTGG GGAGGTCAAC
4800
TCTTGAAGGT GTTGCGGTTC CTGATGTATT TTGCGCTGTG ACCTCTTTGG GTATTATCAC
4860
CTTTCCTTGT CTCTCGGGTC CCTATAGGTC CCTTGAGTTC TCTAACCAGC ACCTCTGGGC
4920
TTCAAGGCCT TTCCCCTCCC ACCTGTAGCT GAAGAGTTTC CGAGTTGAAA GGGCACGGAA
4980
AGCTAAGTGG GAAAGGAGGT TGCTGGACCC AGCAGCAAAA CCCTACATTC TCCTTGTCTC
5040
TGCCTCGGAG CCATTGAACA GCTGTGAACC ATGCCTCCCT CAGCCTCCTC CCACCCCTTC
5100
CTGTCCTGCC TCCTCATCAC TGTGTAAATA ATTTGCACCG AAATGTGGCC GCAGAGCCAC
5160
GCGTTCGGTT ATGTAAATAA AACTATTTAT TGTGCTGGGT TCCAGCCTGG GTTGCAGAGA
5220
CCACCCTCAC CCCACCTCAC TGCTCCTCTG TTCTGCTCGC CAGTCCTTTT GTTATCCGAC
5280
CTTTTTTCTC TTTTACCCAG CTTCTCATAG GCGCCCTTGC CCACCGGATC AGTATTTCCT
5340
TCCACTGTAG CTATTAGTGG CTCCTCGCCC CCACCAATGT AGTATCTTCC TCTGAGGAAT
5400
AAAATATCTA TTTTTATCAA CGACTCTGGT CCTTGAATCC AGAACACAGC ATGGCTTCCA
5460
ACGTCCTCTT CCCTTCCAAT GGACTTGCTT CTCTTCTCAT AGCCAAACAA AAGAGATAGA
5520
GTTGTTGAAG ATCTCTTTTC CAGGGCCTGA GCAAGGACCC TGAGATCCTG ACCCTTGGAT
5580
GACCCTAAAT GAGACCAACT AGGGATC
5607

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2301 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
AGCAGAGCGG ACGGGCGCGC GGGAGGCGCG CAGAGCTTTC GGGCTGCAGG CGCTCGCTGC
60
CGCTGGGGAA TTGGGCTGTG GGCGAGGCGG TCCGGGCTGG CCTTTATCGC TCGCTGGGCC
120
CATCGTTTGA AACTTTATCA GCGAGTCGCC ACTCGTCGCA GGACCGAGCG GGGGGCGGGG
180
GCGCGGCGAG GCGGCGGCCG TGACGAGGCG CTCCCGGAGC TGAGCGCTTC TGCTCTGGGC
240
ACGCATGGCG CCCGCACACG GAGTCTGACC TGATGCAGAC GCAAGGGGGT TAATATGAAC
300
GCCCCTCTCG GTGGAATCTG GCTCTGGCTC CCTCTGCTCT TGACCTGGCT CACCCCCGAG
360
GTCAACTCTT CATGGTGGTA CATGAGAGCT ACAGGTGGCT CCTCCAGGGT GATGTGCGAT
420
AATGTGCCAG GCCTGGTGAG CAGCCAGCGG CAGCTGTGTC ACCGACATCC AGATGTGATG
480
CGTGCCATTA GCCAGGGCGT GGCCGAGTGG ACAGCAGAAT GCCAGCACCA GTTCCGCCAG
540
CACCGCTGGA ATTGCAACAC CCTGGACAGG GATCACAGCC TTTTTGGCAG GGTCCTACTC
600
CGAAGTAGTC GGGAATCTGC CTTTGTTTAT GCCATCTCCT CAGCTGGAGT TGTATTTGCC
660
ATCACCAGGG CCTGTAGCCA AGGAGAAGTA AAATCCTGTT CCTGTGATCC AAAGAAGATG
720
GGAAGCGCCA AGGACAGCAA AGGCATTTTT GATTGGGGTG GCTGCAGTGA TAACATTGAC
780
TATGGGATCA AATTTGCCCG CGCATTTGTG GATGCAAAGG AAAGGAAAGG AAAGGATGCC
840
AGAGCCCTGA TGAATCTTCA CAACAACAGA GCTGGCAGGA AGGCTGTAAA GCGGTTCTTG
900
AAACAAGAGT GCAAGTGCCA CGGGGTGAGC GGCTCATGTA CTCTCAGGAC ATGCTGGCTG
960
GCCATGGCCG ACTTCAGGAA AACGGGCGAT TATCTCTGGA GGAAGTACAA TGGGGCCATC
1020
CAGGTGGTCA TGAACCAGGA TGGCACAGGT TTCACTGTGG CTAACGAGAG GTTTAAGAAG
1080
CCAACGAAAA ATGACCTCGT GTATTTTGAG AATTCTCCAG ACTACTGTAT CAGGGACCGA
1140
GAGGCAGGCT CCCTGGGTAC AGCAGGCCGT GTGTGCAACC TGACTTCCCG GGGCATGGAC
1200
AGCTGTGAAG TCATGTGCTG TGGGAGAGGC TACGACACCT CCCATGTCAC CCGGATGACC
1260
AAGTGTGGGT GTAAGTTCCA CTGGTGCTGC GCCGTGCGCT GTCAGGACTG CCTGGAAGCT
1320
CTGGATGTGC ACACATGCAA GGCCCCCAAG AACGCTGACT GGACAACCGC TACATGACCC
1380
CAGCAGGCGT CACCATCCAC CTTCCCTTCT ACAAGGACTC CATTGGATCT GCAAGAACAC
1440
TGGACCTTTG GGTTCTTTCT GGGGGGATAT TTCCTAAGGC ATGTGGCCTT TATCTCAACG
1500
GAAGCCCCCT CTTCCTCCCT GGGGGCCCCA GGATGGGGGG CCACACGCTG CACCTAAAGC
1560
```

CTACCCTATT CTATCCATCT CCTGGTGTTC TGCAGTCATC TCCCCTCCTG GCGAGTTCTC
1620
TTTGGAAATA GCATGACAGG CTGTTCAGCC GGGAGGGTGG TGGGCCCAGA CCACTGTCTC
1680
CACCCACCTT GACGTTTCTT CTTTCTAGAG CAGTTGGCCA AGCAGAAAAA AAAGTGTCTC
1740
AAAGGAGCTT TCTCAATGTC TTCCCACAAA TGGTCCCAAT TAAGAAATTC CATACTTCTC
1800
TCAGATGGAA CAGTAAAGAA AGCAGAATCA ACTGCCCCTG ACTTAACTTT AACTTTTGAA
1860
AAGACCAAGA CTTTTGTCTG TACAAGTGGT TTTACAGCTA CCACCCTTAG GGTAATTGGT
1920
AATTACCTGG AGAAGAATGG CTTTCAATAC CCTTTTAAGT TTAAAATGTG TATTTTTCAA
1980
GGCATTTATT GCCATATTAA AATCTGATGT AACAAGGTGG GGACGTGTGT CCTTTGGTAC
2040
TATGGTGTGT TGTATCTTTG TAAGAGCAAA AGCCTCAGAA AGGGATTGCT TTGCATTACT
2100
GTCCCCTTGA TATAAAAAAT CTTTAGGGAA TGAGAGTTCC TTCTCACTTA GAATCTGAAG
2160
GGAATTAAAA AGAAGATGAA TGGTCTGGCA ATATTCTGTA ACTATTGGGT GAATATGGTG
2220
GAAAATAATT TAGTGGATGG AATATCAGAA GTATATCTGT ACAGATCAAG AAAAAAAGGA
2280
AGAATAAAAT TCCTATATCA T
2301

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2814 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCATGT CTTACGGTCA AGGCAGAGGG CCCAGCGCCA CTGCAGCCGC GCCACCTCCC
60
AGGGCCGGGC CAGCCCAGGC GTCCGCGCTC TCGGGGTGGA CTCCCCCCGC TGCGCGCTCA
120
AGCCGGCGAT GGCTCCTCTC GGATACCTCT TAGTGCTCTG CAGCCTGAAG CAGGCTCTGG
180
GCAGCTACCC GATCTGGTGG TCCTTGGCTG TGGGACCCCA GTACTCCTCT CTGAGCACTC
240
AGCCCATTCT CTGTGCCAGC ATCCCAGGCC TGGTACCGAA GCAGCTGCGC TTCTGCAGGA
300
ACTACGTGGA GATCATGCCC AGCGTGGCTG AGGGTGTCAA AGCGGGCATC CAGGAGTGCC
360
AGCACCAGTT CCGAGGCCGG CGTTGGAACT GCACCACCGT CAGCAACAGC CTGGCCATCT
420
TTGGCCCTGT TCTGGACAAA GCCACCCGGG AGTCAGCCTT TGTCCATGCC ATCGCCTCCG
480
CTGGAGTAGC TTTCGCAGTG ACACGCTCCT GTGCAGAGGG ATCAGCTGCT ATCTGTGGGT
540
GCAGCAGCCG CCTCCAGGGC TCCCCAGGCG AGGGCTGGAA GTGGGGCGGC TGTAGTGAGG
600
ACATTGAATT TGGAGGAATG GTCTCTCGGG AGTTTGCCGA TGCCAGGGAG AACCGGCCGG
660
ATGCCCGCTC TGCCATGAAC CGTCACAACA ATGAGGCTGG GCGCCAGGCC ATCGCCAGTC
720

ACATGCACCT CAAGTGCAAA TGCCACGGGC TATCTGGCAG CTGTGAAGTG AAGACCTGCT
780
GGTGGTCGCA GCCGGACTTC CGCACCATCG GGGATTTCCT CAAGGACAAG TATGACAGTG
840
CCTCGGAGAT GGTGGTAGAG AAACACCGAG AGTCTCGTGG CTGGGTGGAG ACCCTGAGGC
900
CACGTTACAC GTACTTCAAG GTGCCGACAG AACGCGACCT GGTCTACTAC GAGGCCTCAC
960
CCAACTTCTG CGAACCTAAC CCCGAAACCG GCTCCTTCGG GACGCGTGAC CGCACCTGCA
1020
ATGTGAGCTC GCATGGCATA GATGGGTGCG ACCTGTTGTG CTGCGGGCGC GGGCATAACG
1080
CGCGCACTGA GCGACGGAGG GAGAAATGCC ACTGTGTTTT CCATTGGTGC TGCTACGTCA
1140
GCTGCCAGGA GTGCACACGT GTCTATGACG TGCACACCTG CAAGTAGGAG AGCTCCTAAC
1200
ACGGGAGCAG GGTTCATTCC GAGGGGCAAG GTTCCTACCT GGGGGCGGGG TTCCTACTTG
1260
GAGGGGTCTC TTACTTGGGG ACTCGGTTCT TACTTGAGGG CGGAGATCCT ACCTGTGAGG
1320
GTCTCATACC TAAGGACCCG GTTTCTGCCT TCAGCCTGGG CTCCTATTTG GGATCTGGGT
1380
TCCTTTTTAG GGGAGAAGCT CCTGTCTGGG ATACGGGTTT CTGCCCGAGG GTGGGGCTCC
1440
ACTTGGGGAT GGAATTCCAA TTTGGGCCGG AAGTCCTACC TCAATGGCTT GGACTCCTCT
1500
CTTGACCCGA CAGGGCTCAA ATGGAGACAG GTAAGCTACT CCCTCAACTA GGTGGGGTTC
1560
GTGCGGATGG GTGGGAGGGG AGAGATTAGG GTCCCTCCTC CCAGAGGCAC TGCTCTATCT
1620
AGATACATGA GAGGGTGCTT CAGGGTGGGC CCTATTTGGG CTTGAGGATC CCGTGGGGGC
1680
GGGGCTTCAC CCCGACTGGG TGGAACTTTT GGAGACCCCC TTCCACTGGG GCAAGGCTTC
1740
ACTGAAGACT CATGGGATGG AGCTCCACGG AAGGAGGAGT TCCTGAGCGA GCCTGGGCTC
1800
TGAGCAGGCC ATCCAGCTCC CATCTGGCCC CTTTCCAGTC CTGGTGTAAG GTTCAACCTG
1860
CAAGCCTCAT CTGCGCAGAG CAGGATCTCC TGGCAGAATG AGGCATGGAG AACAACTCAG
1920
GGGTGATACC AAGACCTAAC AAACCCCGTG CCTGGGTACC TCTTTTAAAG CTCTGCACCC
1980
CTTCTTCAAG GGCTTTCCTA GTCTCCTTGG CAGAGCTTTC CTGAGGAAGA TTTGCAGTCC
2040
CCCAGAGTTC AAGTGAACAC CCATAGAACA GAACAGACTC TATCCTGAGT AGAGAGGGTT
2100
CTCTAGGAAT CTCTATGGGG ACTGCTAGGA AGGATCCTGG GCATGACAGC CTCGTATGAT
2160
AGCCTGCATC CGCTCTGACA CTTAATACTC AGATCTCCCG GGAAACCCAG CTCATCCGGT
2220
CCGTGATGTC CATGCCCCAA ATGCCTCAGA GATGTTGCCT CACTTTGAGT TGTATGAACT
2280
TCGGAGACAT GGGGACACAG TCAAGCCGCA GAGCCAGGGT TGTTTCAGGA CCCATCTGAT
2340
TCCCCAGAGC CTGCTGTTGA GGCAATGGTC ACCAGATCCG TTGGCCACCA CCCTGTCCCG
2400
AGCTTCTCTA GTGTCTGTCT GGCCTGGAAG TGAGGTGCTA CATACAGCCC ATCTGCCACA
2460
AGAGCTTCCT GATTGGTACC ACTGTGAACC GTCCCTCCCC CTCCAGACAG GGGAGGGGAT
2520
GTGGCCATAC AGGAGTGTGC CCGGAGAGCG CGGAAAGAGG AAGAGAGGCT GCACACGCGT
2580

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-13

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08716

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-13, drawn to a population of mammalian neural precursor cells committed to a cell fate.

Group II, claim(s) 14-16, drawn to a method of stimulating proliferation of a heterogenous population of neural cell precursor cells to enrich for dorsal neural cells.

Group III, claim(s) 17-18 and 20, drawn to a method of inducing neuronal regeneration in an adult mammal comprising transplanting dorsal neural precursor cells.

Group IV, claim(s) 19, drawn to a method of inducing neuronal regeneration in an adult mammal comprising administering a Wnt polypeptide or Wnt agonist.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is directed to a population of mammalian neural precursor cells, which is the first product. However, because Boss et al teach an enriched population of porcine or human neuron progenitor cells (i.e., mammalian neural precursor cells), no special technical feature exists for Group I as defined by PCT RULE 13.2, because it does not define a contribution over the prior art. The technical features of Groups II-IV are drawn to methods having different goals, method steps and starting materials, which do not share the same or a corresponding technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application. Because the technical feature of Group I is not a special technical feature, and because the technical features of the Group II-IV inventions are not present in the Group I claims, unity of invention is lacking.